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Aminoacyl-tRNA Binding Activity in Regenerating Kidney following Contralateral Nephrectomy or Administration of Folic Acid

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Homogenates of kidney cortex obtained 2 or 7 days following contralateral nephrectomy or folic acid injection were separated into a ribosome fraction and into a postmicrosomal pH 5 supernatant fraction. The activity of each of these fractions for peptide synthesis from [^{14}C]phenylalanyl-tRNA was tested by complementation with similar fractions obtained from normal rat liver. While the ribosomal activity of regenerating kidney was not changed, there was an increase in the activity of the pH 5 supernatant fraction obtained from regenerating kidney. The results provided evidence that the increased incorporation observed with the pH 5 supernatant fraction obtained from regenerating kidney was due to an increased activity of elongation factor 1.

INTRODUCTION

Hypertrophy and hyperplasia of the kidney cortex of young rats following the administration of growth hormone or the aminonucleoside derivative of puromycin (AN) has been shown to involve an increase in the ability of the pH 5 supernatant fraction (freed of tRNA and aminoacyl synthetases) to support protein synthesis. Relatively greater amounts of elongation factor 2 (EF 2) rather then elongation factor 1 (EF 1) are present in this soluble cytoplasmic fraction, and the increased activity of this fraction during such growth was shown to be due to an increased ability of elongation factor 1 to promote the factor-dependent binding of aminoacyl-RNA to ribosomes (Girgis and Nicholls, 1971, 1972, 1973). While growth following growth hormone is believed to involve the synthesis of numerous proteins, hypertrophy following AN particularly involves the increased synthesis of a soluble ribonuclease inhibitor protein (Nicholls and Markle, 1974; Bishay and Nicholls, 1973). The latter protein may serve a protective function in this and in other situations where increased amounts of ribonuclease pass into the glomerular filtrate (Rosso *et al.*, 1973).

A number of experimental techniques have been used to study induced kidney growth (Nowinski and Goss, 1969), and this growth has been correlated with increases in DNA, RNA and protein content (reviewed by Halliburton (1969) and by Bucher and Malt, (1971)). Following contralateral nephrectomy, measurement of the incorporation of labeled precursors into RNA have recently led to the conclusion that there may be a decreased degradation of newly synthesized RNA as well as other changes in RNA metabolism (Malt and Lemaitre, 1968; Willems *et al.*, 1969; Hill *et al.*, 1974; Hill, 1975). Renal growth following folic acid administration is greater than that following contralateral nephrectomy and, while it may involve additional features, it is characterized by marked increases in DNA, RNA and protein content (Threlfall, 1969; Taylor *et al.*, 1968; Baserga *et al.*, 1968; Oertel and Herken, 1974).

Studies of labeled amino acid incorporation into protein carried out *in vivo* and in renal slices have supported the view that the regenerating kidney may well have an increased rate of protein synthesis together with an increased labeling of intracellular free amino acid pools (Coe and

Korty, 1967; Tomashefsky and Tannenbaum, 1969; Threlfall, 1969). Because of the difficulty in identifying and measuring *in vivo* the specific activities of precursor pools for protein synthesis (Mortimore *et al.*, 1972; Airhart *et al.*, 1974), the present experiments, with cell-free preparations of kidney and charged aminoacyl-tRNA, were carried out during growth of kidney in response to contralateral nephrectomy or folic acid administration.

MATERIALS AND METHODS

Animals. Young male Sprague-Dawley rats (120–130 g) were used and had free access to food (Master Fox Cubes) and water throughout the experiment. Animals that were subjected to left unilateral nephrectomy, sparing the adrenal (operated) or to sham operation (sham) were anaesthetized with Nembutal by the intraperitoneal injection of 0.4 mg/100 g body weight and this was supplemented by light ether anaesthetic. Following the operation, the subdermal tissue was sutured with 000 cuticular silk (Look, Inc., Boston) and the skin was drawn together with 9-mm stainless-steel wound clips (Clay-Adams, Inc., New York). For sham nephrectomy the left kidney was exposed for a similar period of time. Rats received folic acid in 0.3 *M* sodium carbonate at 25 mg/100 g body weight by intraperitoneal injection.

Chemicals. Uniformly labeled L-[^{14}C]phenylalanyl-tRNA (specific activity 450 mCi/mmol) was prepared from control rat liver by the method of Moldave (1963). The preparation contained 19 unlabeled aminoacyl-tRNA species and had a specific activity of 120 dpm/μg of RNA. For the binding experiments, uniformly labeled *Escherichia coli* [^{14}C]phenylalanyl-tRNA with a specific activity of 370 dpm/μg of RNA obtained from New England Nuclear was used. Sodium fusidate was a gift from the Squibb Institute for Medical Research, New Brunswick, N. J., and from Leo Pharmaceutical Products, Copenhagen. The ammonium salt of aurintricarboxylic acid (ATA), the sodium salt of GTP, dithiothreitol (DDT), and folic acid were obtained from Sigma Chemicals.

Kidney homogenate. The right kidney was removed to ice and all subsequent preparation was carried out at 2–4°C. The medulla was removed by dissection, the kidneys weighed and homogenized in a glass homogenizer with eight passes of a Teflon pestle in nine volumes of 0.25 *M* sucrose in Buffer I composed of 50 m*M* Tris–HCl buffer (pH 7.8 at 25°C), 80 m*M* KCl, 6 m*M* $MgCl_2$, and 10 m*M* mercaptoethanol. The homogenate was centrifuged at 15,000*g* for 15 min in a Spinco Model L2-65 ultracentrifuge. The postmitochondrial supernatant fraction was centrifuged for 1 hr at 105,000*g* to sediment the microsomes which were retained for preparation of ribosomes. The postmicrosomal supernatant (S105) fraction was reserved for preparation of the pH 5 supernatant fraction.

Kidney ribosomes. The microsomes were resuspended in 0.25 *M* sucrose in Buffer I containing a final concentration of 1% sodium deoxycholate and were stirred for 10 min in the cold, prior to layering over 3 ml of 0.5 *M* sucrose in Buffer I which in turn was layered over 3 ml of 1.0 *M* sucrose in Buffer I. Following centrifugation at 105,000*g* for 120 min, the supernatant layers were carefully aspirated and the pellet was rinsed in Buffer I and resuspended in 0.25 *M* sucrose in Buffer I containing 15% glycerol. The ribosomes were stored at a concentration of 1 mg of RNA/ml at −20°C.

Kidney and liver pH 5 supernatant fraction. The S105 fraction of the kidney homogenate and of a 20% liver homogenate prepared in the same way was freed of synthetases and RNA, including tRNA, by adjusting the pH to 5.2 with 1 *M* acetic acid. After standing for 1 hr, the precipitate was removed by centrifugation at 15,000*g* for 10 min. The supernatant fluid was brought to a pH of 7.8 by the addition of 1 *M* KOH. This resultant pH 5 supernatant fraction, containing a final concentra

ion of 15% glycerol, was stored at −20°C.

Preincubated, salt-washed control liver ribosomes. Microsomes were prepared rom 20% homogenates of liver of control ats by the same method as described bove for kidney microsomes and were sed to prepare preincubated, salt-washed ver ribosomes as described previously (Nicholls, 1973).

Preparation of liver EF 1 and EF 2. The H 5 supernatant fraction of control rat ver was obtained and elongation factor 1 repared as described previously (Girgis nd Nicholls, 1973) using Sephadex G-200 el-filtration chromatography followed by ydroxylapatite chromatography. Elongaion factor 2 was prepared by DEAE-cellulose chromatography as described earlier Girgis and Nicholls, 1973). The EF 1 and F 2 preparations were tested separately r [^{14}C]phenylalanyl-tRNA incorporation nto peptide. The incorporation in both ases was less than 5% of that observed hen the same reaction mixture contained oth EF 1 and EF 2. No incorporation was bserved when [^{14}C]phenylalanine was sed instead of [^{14}C]phenylalanyl-tRNA.

Measurement of protein and RNA. Hot % trichloroacetic acid-insoluble protein as prepared for counting as described by Nicholls *et al.* (1970), and the counting fficiency was 85%. Incorporation is expressed as disintegrations per minute per illigram of ribosomal RNA. These figres were corrected by subtraction of vales for nonincubated tubes which were approximately 70 dpm/tube. Protein concenration was determined by the method of owry *et al.* (1953) or by the method of ombs *et al.* (1959) when high concentraions of dithiothreitol and mercaptoethaol interfered with the Lowry method. NA was estimated from the extinction at 60 nm ($E^{1\%}_{1cm}$ = 230). The results given in he figures are mean values of duplicate ubes representative of those obtained in a umber of separate experiments. The eans ± SEM are shown on the graphs or n the legends.

RESULTS

The wet weight of the regenerating right kidney increased 18%, 2 days following the operation, and 32%, 7 days following the operation (Fig. 1), with no change in the proportion of wet to dry weight (not shown), in confirmation of previous work (reviewed by Halliburton (1969) and by Bucher and Malt (1971)). Table 1 shows that the concentration of protein in the postmicrosomal supernatant and in the pH 5 supernatant fractions was not significantly changed at 2 or 7 days after the operation, while the ribosomal RNA fraction was significantly increased at these times. Thus, the protein content per kidney of the fractions studied is increased proportional to the kidney weight, while the rRNA content per kidney of the ribosome fraction studied is increased disproportionately, as previously described (Malt, 1969).

The ribosome fraction, isolated as described, did not exhibit an increase in the ability to incorporate [^{14}C]phenylalanyl-tRNA into peptide, either using endoge-

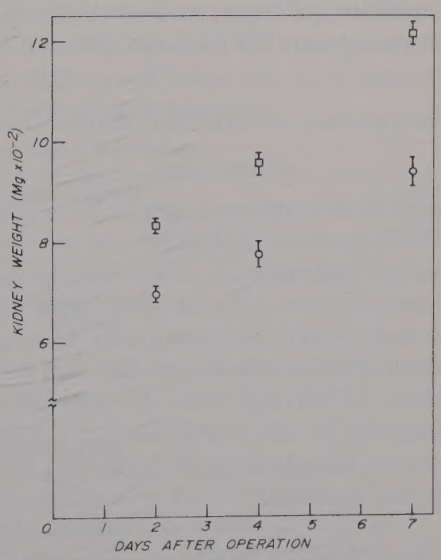

FIG. 1. The weight of the right kidney cortex of operated and sham animals. The means ± SEM are shown at 2, 4 and 7 days following the operation for ten, five and nine animals, respectively. (□), Regenerating; (○), sham.

nous mRNA or exogenous poly(U), incubated together with a pH 5 supernatant fraction derived from normal control rat liver. The result was the same with the ribosome fraction obtained 2 days after operation (Fig. 2) as it was 7 days after operation (not shown). Thus, although the concentration and kidney content of ribosomes is increased, the specific activity is apparently unchanged.

The results obtained when the pH 5 supernatant fraction of regenerating kidney was incubated with control liver ribosomes were markedly different, since 2 days after the operation there was a significant increase (approximately 40%) in the ability of this fraction to support the incorporation of [^{14}C]phenylalanyl-tRNA into polypeptide chains (Fig. 3a). The increase was no longer evident 7 days after the operation (Fig. 3b) but, since the protein content per kidney was significantly elevated at both times, the total activity (per kidney) of the pH 5 supernatant was increased not only 2 days but also 7 days following the operation. Similar results were found using incubation times shorter than 10 min. The addition of high concentrations of dithiothreitol or GTP did not remove the difference between the pH 5 supernata fraction activity of 2-day regenerating ki ney and the control preparation (Figs. and b), as might be expected if a thiol- an GTP-sensitive inhibitor were present the control kidney as it is in the case control liver (Scornik *et al.*, 1967; Nola and Hoagland, 1971). Furthermore the a dition of increasing amounts of poly(U (Fig. 4c) did not remove the increased acti ity, as might be expected if differenti amounts of ribonuclease activity were r sponsible for the effects observed at 2 day In other experiments the increased acti ity was still found when either ribosom or phenylalanyl-tRNA were increase threefold. Thus the changes in ribon clease and inhibitor-bound ribonuclea (Rosso *et al.*, 1973) do not account for t increased activity of the pH 5 supernata fraction.

Since EF 1 and EF 2 were present in t pH 5 supernatant fraction and not on t salt-washed control liver ribosomes, it w desirable to see whether the activity either of these factors was increased in t kidney pH 5 supernatant fraction of the day regenerating kidney. When the pH supernatant fractions from the 2-day r

TABLE 1

SOLUBLE PROTEIN AND RIBOSOMAL RNA CONCENTRATION IN HOMOGENATE FRACTIONS OF KIDNEY DURIN GROWTH FOLLOWING UNILATERAL NEPHRECTOMY[a]

Days after operation	Protein (mg/100 g wet weight)				*P*	
	Operated		Sham		S105	pH
	S105 fraction	pH 5 supernatant fraction	S105 fraction	pH 5 supernatant fraction		
2	5.36 ± 0.43 (7)	3.65 ± 0.07 (8)	5.04 ± 0.47 (7)	3.39 ± 0.13 (9)	> 0.2	> 0.1
7	5.01 ± 0.16 (5)	3.75 ± 0.07 (9)	4.50 ± 0.15 (5)	3.68 ± 0.15 (8)	> 0.05	> 0.2
	RNA (mg/100 g wet weight)					
	rRNA		rRNA		*P*	
2	0.132 ± 0.001 (5)		0.116 ± 0.007 (5)		< 0.05	
7	0.142 ± 0.001 (5)		0.111 ± 0.005 (4)		< 0.01	

[a] The fractions were prepared and the protein and RNA determined as described under Materials an Methods. Numbers given are means ± SEM. The numbers of animals used are in parentheses. *P* value according to Student's *t* test.

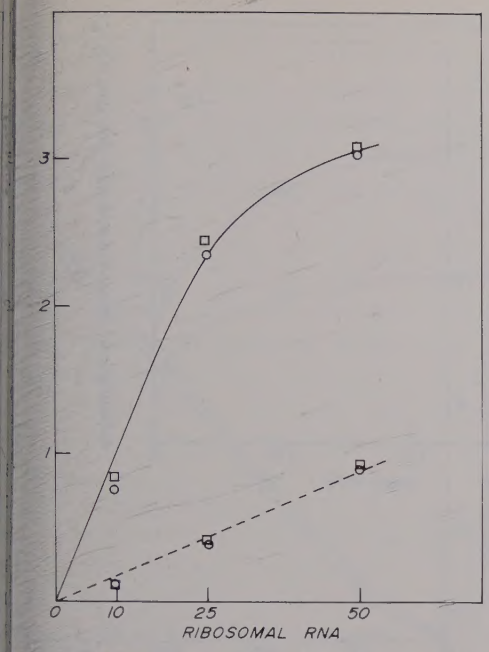

FIG. 2. The incorporation of [^{14}C]phenylalanyl-NA into peptide, when ribosomes (60 μg of RNA) m the kidney of operated or sham rats 2 days lowing the operation were incubated with 600 μg pH 5 supernatant protein from control rat liver for min at 37°C in 0.40 ml medium containing 60 m*M* is–HCl (pH 7.8 at 25°C), 6 m*M* $MgCl_2$, 0.2 m*M* TP, [^{14}C]phenylalanyl-tRNA (10,000 dpm, 80 μg of NA) and 100 μg of poly(U) were added. (□– –□), generating with endogenous mRNA; (○– –○), am with endogenous mRNA; (□——□), regenerat-g with poly(U), (○——○), sham with poly(U).

nerating kidney and from the sham con-ol kidney were supplemented with in-easing amounts of control liver EF 2 ig. 5a), the results made it unlikely that tered amounts of EF 2 are responsible. o significant increase in [^{14}C]phenyl-anyl-tRNA incorporation occurred, when F 2 was added either to the pH 5 super-atant fraction from sham kidney or from e 2-day regenerating kidney. Thus ex-ss EF 2 is present in both these prepara-ons.

Similar experiments were carried out by upplementing the reaction mixture with ntrol liver EF 1 (Fig. 5b). The addition of) μg of protein of liver EF to the incuba-on mixture containing pH 5 supernatant action from sham kidney increased the incorporation of [^{14}C]phenylalanyl-tRNA to a level higher than that seen with the unsupplemented pH 5 supernatant fraction from the 2-day regenerating kidney, showing that kidney EF 1 was limiting in the sham preparation. Since EF 1 catalyzed the poly(U)-directed binding of [^{14}C]phenylalanyl-tRNA to ribosomes at low Mg^{2+} concentrations, the binding activity was measured as previously described (Malkin and Lipmann, 1969). The stimulation of binding by the pH 5 supernatant fraction from 2-day regenerating kidney was 30% greater than that by the pH 5 supernatant fraction from sham kidney (Fig. 6).

Figures 7a and b show that the results obtained with the pH 5 supernatant fraction obtained from kidney of rats treated 2 days earlier with folic acid were in the same direction as, though considerably greater than, those obtained from 2-day regenerating kidney. The activity of the pH 5 supernatant fraction was markedly increased above the control preparation at 2 days (though not 6 days) following folic acid administration. The supplementation of the control kidney preparation with purified liver EF 1 raised the incorporation of phenylalanyl-tRNA to the level seen with the folic acid preparation.

DISCUSSION

From all of these results it is clear that there is an increased aminoacyl-tRNA binding activity in regenerating kidney 2 days after contralateral nephrectomy or folic acid administration. There is no evidence that this increase is accompanied by an increase in the activity of soluble mRNA binding factors nor of elongation factor 2 activity which is already in excess over elongation factor 1 (Girgis and Nicholls, 1972).

The results show that the specific activity of ribosomes in the translation of endogenous mRNA or poly(U) is unchanged in regenerating kidney. While it is possible that the presence of increased amounts of

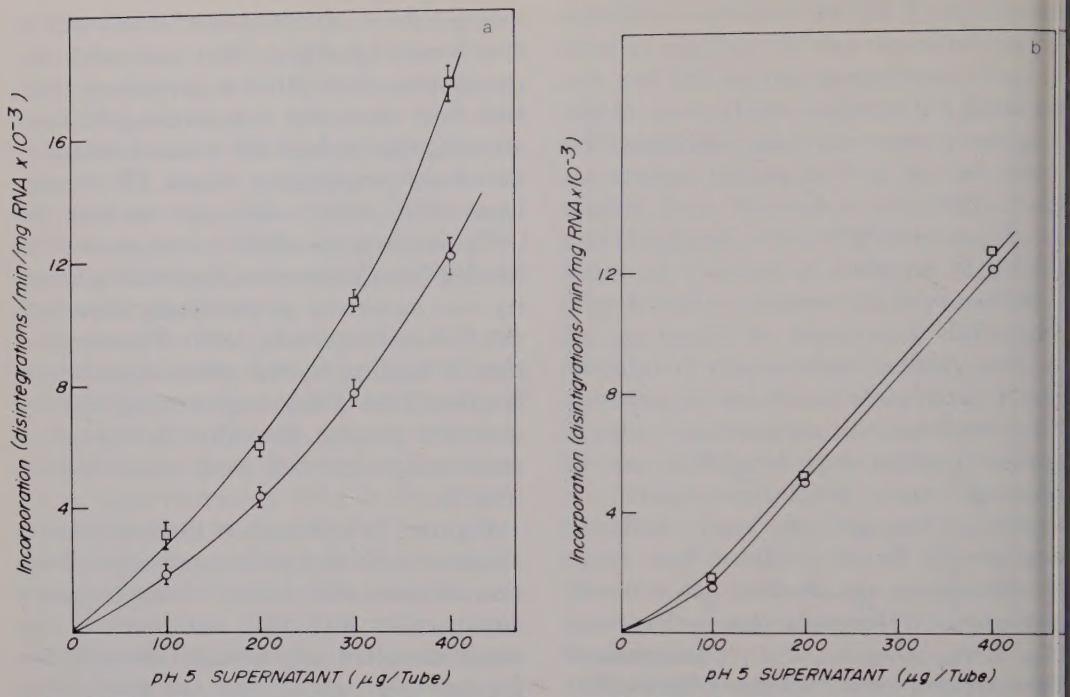

FIG. 3. The effect of incubating pH 5 supernatant fraction obtained from the kidney of sham or operated rats with excess salt-washed ribosomes (60 μg of RNA) from the liver of control rats. These ribosomes were preincubated to remove endogenous mRNA and the incorporation was dependent upon the addition of poly U. Conditions as described for Fig. 2. (a), Two days following the operation. Mean ± SEM for seven rats. $P < 0.01$, except for 100 μg where $0.05 < P < 0.10$. (b), Seven days following the operation. Means ± SEM for five rats at 100 μg, 200 μg and 400 μg were 1720 ± 146, 5140 ± 330 and 12,800 ± 640 dpm for operated rats and 1522 ± 224, 5000 ± 660 and 12,100 ± 1560 dpm for sham rats, respectively, with no statistically significant differences. (□——□), Regenerating supernatant; (○——○), sham supernatant fraction.

ribonuclease, as well as its inhibitor, in regenerating kidney (Rosso *et al.*, 1973) may promote increased degradation of endogenous mRNA during the isolation of kidney ribosomes, it appears that ribosomal activity is intact. Attempts to measure the amounts of mRNA that is free or that is bound to ribosomes will require special precautions to remove the influence of ribonuclease activity. The increase in kidney ribosomal concentration is an important feature of increased translation in regenerating kidney (Malt, 1969).

It has been observed that aldosterone and other steroids increase kidney ribosomal activity and change the phosphorylation of ribosomal proteins (Trachewsky *et al.*, 1972; Hill and Trachewsky, 1974), but such a change in ribosomal activity was not detected in the present experiments on regenerating kidney. As in kidney stimulated by aldosterone, modifications of the ribosomal proteins also occur in liver regenerating after partial hepatectomy (Liew and Gornal, 1973; Gressner and Wool, 1974; Scheinbuks *et al.*, 1974; Anderson *et al.*, 1975). However, it has been reported that there is no change in the rate of translation by polyribosomes studied *in vivo* in regenerating liver (Scornik, 1974a, b), in agreement with earlier results on ribosomes purified from the microsomal fraction (Scornik *et al.*, 1967). It seems likely that at least part of the increased protein content of regenerating liver is a result of a reduced rate of degradation (Swick and Ip, 1974; Hill and Malamud, 1974) coupled with increased polysome aggregation (Rizzo and Webb, 1968).

Recent studies of the rate of degradation

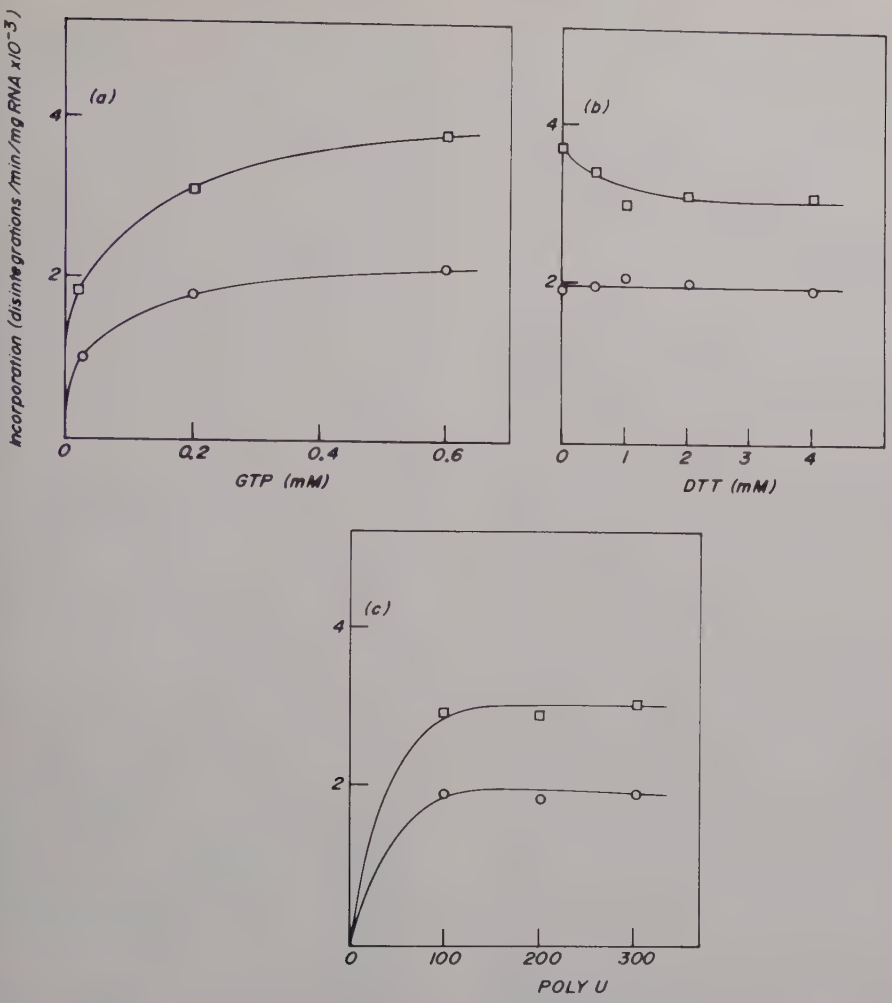

FIG. 4. The effect of varying (a) GTP, (b) dithiothreitol or (c) poly(U) concentrations, on the incorporation of [^{14}C]phenylalanyl-tRNA into peptide with pH 5 supernatant fraction from operated or sham rats 2 days following the operation. The incubation was carried out for 10 min as described for Fig. 3 using preincubated control liver ribosomes and poly(U) together with 100 μg of kidney pH 5 supernatant protein from regenerating (□——□) or sham (○——○) animals.

of proteins in regenerating kidney and in other tissues during stimulated growth have been carried out by Hill and Malamud (1974). They found that the mean half-life of kidney proteins, as measured by the loss of protein radioactivity following the injection of [^{14}C]guanido-labeled arginine, was increased 66% during compensatory renal hypertrophy. They have calculated that more than 95% of the net protein gain may be accounted for by a decrease in protein catabolism. Previous studies of the incorporation of labeled amino acids into protein in kidney following contralateral nephrectomy or folic acid administration have been carried out *in vivo* where there are difficulties in identifying and measuring the specific activity of the precursor pool for protein synthesis (Mortimore *et al.*, 1972; Airhart *et al.*, 1974). Thus, while an increased incorporation was found *in vivo* or in renal slices of regenerating kidney (Coe and Korty, 1967; Tomashefsky and Tannenbaum, 1969; Threlfall, 1969), it is difficult to obtain an estimate of the rate of synthesis. In the

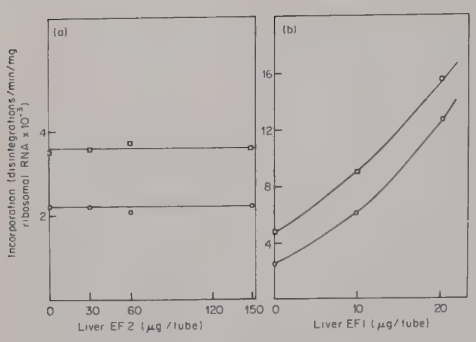

FIG. 5. Incorporation of [^{14}C]phenylalanyl-tRNA into peptide using 100 μg of kidney pH 5 supernatant protein obtained 2 days after operation supplemented with (a) control liver elongation factor 2 and (b) control liver elongation factor 1 at the concentrations indicated. Incubation as for Fig. 3 using preincubated control liver ribosomes and poly(U). (□——□), Regenerating; (○——○), sham animals.

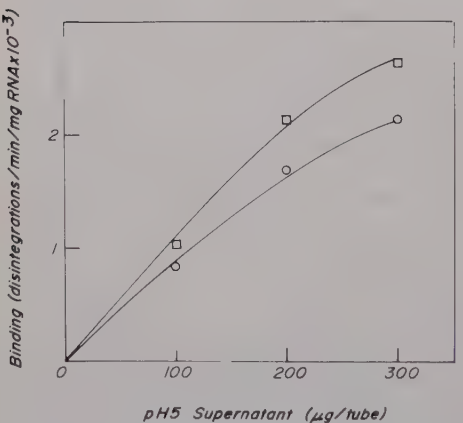

FIG. 6. The poly(U)-dependent binding of [^{14}]phenylalanyl-tRNA to preincubated, salt-washed control liver ribosomes (300 μg of RNA) using kidney pH 5 supernatant protein obtained 2 days after operation. The incubation was in a volume of 0.4 ml for 8 min at 37°C as described for Fig. 3 except for 0.4 m*M* GTP and 13,700 dpm of *E. coli* [^{14}C]phenylalanyl-tRNA in the presence of 4 m*M* fusidic acid which inhibits translocation (Malkin and Lipmann, 1969). The reaction was terminated by adding ice-cold buffer and the samples washed with three portions of buffer on Millipore filters (HA, 0.45 μm). The filters were solubilized in 2 ml of NCS solubilizer, diluted with scintillation fluid and counted as described in Materials and Methods. Blanks containing all the reactants except pH 5 supernatant fraction were subtracted. (□——□), regenerating; (○——○), sham animals.

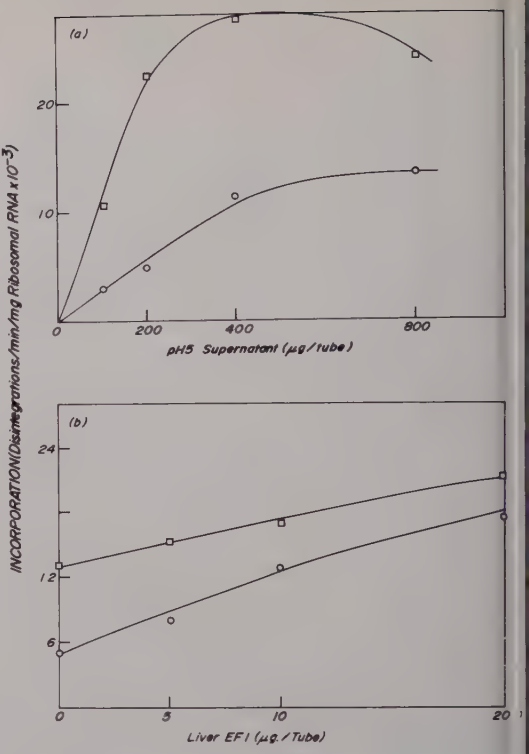

FIG. 7. Incorporation of [^{14}C]phenylalanyl-tRNA into peptide using preincubated control liver ribosomes and poly(U) together with kidney pH 5 supernatant protein obtained 2 days after a single injection of folic acid or from control rats. (a), Effect of concentration of pH 5 supernatant fraction. (b), Kidney pH 5 supernatant (250 μg of protein) was supplemented with control liver elongation factor 1 at the concentrations indicated. (□——□), Folic acid; (○——○), controls.

present experiments the rate of synthesis that can be supported by the cell sap elongation factors has been tested *in vitro* and found to be increased 2 days but not 7 days after the stimuli. Thus it seems likely that the marked decrease in degradation of kidney protein is accompanied by an increase in synthesis. A somewhat similar situation appears to result following insulin administration (Rannels *et al.*, 1975).

In the experiments reported here it is important to stress that liver ribosomes obtained from control animals are used and that the pH 5 supernatant fraction is tested in the presence of excess exogenous mRNA and [^{14}C]phenylalanyl-tRNA. The

evidence obtained strongly supports the view that the binding of this labeled precursor to ribosomes, preparatory to bond synthesis and translocation, is increased due to an increased activity of elongation factor 1. We have observed a similar increase in kidney, though not liver, following growth hormone or aminonucleoside administration, and in both kidney and liver following DDT administration, as well as in liver following laser irradiation (Girgis and Nicholls, 1973, 1971; Nicholls and Girgis, 1970; Cappon and Nicholls, 1974a, b; Nicholls, 1973; Nicholls *et al.*, 1974). Similar observations have been made for spleen of immunized rats (Willis and Starr, 1971) and adrenal following ACTH (Scriba and Reddy, 1965). Thus an increased activity of elongation factor 1 frequently appears to be present in cells which are undergoing hypertrophy. Because of the difficulties in purifying this multimeric protein that has different activity in different states of aggregation (Collins *et al.*, 1972; Moon *et al.*, 1973; Legocki *et al.*, 1974; Drews *et al.*, 1974), it is not yet possible to decide whether the increased activity of elongation factor 1 is due to an increase in amount and/or reactivity. Moreover, the nature of the factor(s) that triggers renal growth following contralateral nephrectomy or folic acid injection is not yet known (for a review, see Malt (1973)).

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Noted added in proof: These conclusions for compensatory renal hypertrophy are in agreement with those of Sendecki *et al.* (1973).

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DNA Synthesis in Unfertilized Sea Urchin Eggs Can Be Turned On and Turned Off by the Addition and Removal of Procaine Hydrochloride

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Unfertilized sea urchin eggs turn on thymidine transport, DNA synthesis and the chromosome cycle in response to procaine hydrochloride. The rates to which these processes activate depend on the extracellular concentration of procaine. Removing procaine turns off DNA synthesis and the chromosome cycle, re-adding procaine turns these processes on once more. Thymidine transport does not turn off after procaine removal. It remains on at the rate it had reached at the time of procaine removal. During a 12-hr period, unfertilized eggs in procaine complete four S-phases, while normal, fertilized embryos complete eight. Tritiated thymidine incorporation into DNA is very low in procaine-treated eggs. This is because in procaine the thymidine transport system is relatively inactive compared to that of fertilized eggs. The data suggest eggs may possess homeostatic mechanisms that actively suppress the DNA synthetic pathway and the chromosome cycle. This system for turning on and off DNA synthesis may prove useful in further analyses of the factors controlling DNA synthesis during early development.

INTRODUCTION

Unfertilized sea urchin eggs are metabolically inactive cells. During the first 30 min following insemination, the sequential activation of many distinct metabolic and synthetic pathways occurs at specific times (Epel *et al.*, 1969, 1974). One of the last of these activations is the initiation of DNA synthesis at 30 min post insemination (Hinegardner *et al.*, 1964).

Knowledge of the mechanism governing initiation of DNA synthesis is central to our understanding of cell replication. Sea urchin eggs are ideal cells for studies of the control of DNA synthesis because they can be obtained in great numbers, they incorporate radioactive precursors into DNA, they are very synchronous, and the period of synthesis (S) is short and very definable in time and by cytological observation (Hinegardner *et al.*, 1964). The study of the control of DNA synthesis would be helped if we had an artificial means to turn on and turn off synthesis at will. This paper described such a system: The unfertilized sea urchin egg treated with the local anesthetic procaine hydrochloride.

While treating eggs with procaine (Vacquier, 1975), we noticed that nuclei disappeared and cytasters formed. The eggs appeared unfertilized; they did not elevate fertilization membranes nor undergo a cortical reaction. Closer observation showed them to possess duplicated mitotic chromosomes. It was assumed therefore that DNA replication had occurred. Labeling experiments showed procaine-treated eggs to be actively synthesizing DNA, RNA and protein. These synthetic activations triggered by procaine closely resemble those in eggs treated with NH_4OH (Loeb, 1913; Mazia, 1974a; Mazia and Ruby, 1974; Epel *et al.*, 1974). After 180–360 min in procaine (2–5 m*M*) unfertilized eggs develop cytasters (Dirksen, 1961) and may undergo cytokinesis. The blastomeres produced are of unequal size, and no attempt was made to raise them as parthenogenetic embryos.

This paper describes the chromosome cycle, DNA synthesis and the activation of

he [^{3}H]TdR[1] transport system in unfertilized eggs treated with procaine. Procaine urns on all three processes at rates proportional to the procaine concentration in the ea water. The surprising finding is that, nce turned on, all three processes turn off when procaine is removed.

MATERIALS AND METHODS

1. Gametes, procaine treatment, culturing conditions and cytological observations. Gametes of *Strongylocentrotus purpuratus* were spawned into sea water by intercoelomic injection of 0.5 *M* KCl. Egg jelly coats were removed by a 2-min exposure to pH 5 sea water followed by two washes in normal sea water. Egg volume was measured by gently sedimenting an aliquot of egg suspension by hand centrifugation (Vacquier and Payne, 1973). Egg suspensions were diluted to 1% (v/v) in normal sea water or sea water containing various millimolar concentrations of procaine hydrochloride (Sigma). Cultures containing 50 mg/liter of penicillin G and streptomycin sulfate were agitated at 15°C on a rocker table at minimum motion. For observations of chromosomes, eggs were fixed 30 min in three changes of ethanol:acetic acid (3:1, v/v) followed by 1 hr in 60% acetic acid. One-hundred eggs were observed with phase contrast microscopy and the number containing condensed chromatin determined. Eggs were scored as containing condensed chromatin beginning at the time when early prophase chromatin threads were clearly visible and ending just before the complete decondensation of the mitotic chromosomes into chromosome vesicles (Hinegardner *et al.*, 1964). The number of chromosomes per egg was not determined for each egg. Eggs were scored as condensed if any chromosomes were visible, regardless of number.

2. Incorporation of [^{3}H]TdR. The procedure used was essentially that described by Hinegardner *et al.* (1964). To measure DNA synthesis in unfertilized eggs treated with procaine, eggs were cultured in sea water containing 1 μCi/ml of [^{3}H]TdR (Schwarz/Mann, 6 Ci/m*M*). At the desired times, two 1-ml samples were removed and added to a 12-ml conical centrifuge tube containing 10 ml of 5% TCA. After 10 min the eggs were sedimented by centrifugation, the supernatant fluid removed by aspiration and the egg pellet dissolved in 0.3 ml of NCS (Amersham/Searle) at 60°C for 20 min and added to a vial containing 10 ml of toluene scintillation cocktail. The radioactivity is expressed as cpm per sample.

[^{3}H]TdR incorporation as measured by this method has been previously proven to be a direct measure of DNA synthesis (Hinegardner *et al.*, 1964). We also found the acid-insoluble radioactivity to be incorporated into DNA on the basis of two criteria. First, 97% of the acid-insoluble cpm were rendered acid-soluble by DNAase I digestion of eggs incubated 240 min in procaine. Second, 100% of the acid-insoluble counts became acid-soluble after extracting the eggs for 15 min in 1 *M* PCA at 95°C.

3. Transport of [^{3}H]TdR. Transport of [^{3}H]TdR into eggs was measured by adding two 1-ml portions of egg suspension to 1 ml of sea water containing 1 μCi of [^{3}H]TdR (6 Ci/m*M*). After 5 min the eggs were sedimented by hand centrifugation, the isotopic sea water aspirated off and the egg pellet resuspended twice in 10 ml of 0°C sea water. The final egg pellet was extracted in 1 ml of cold 5% TCA, centrifuged to sediment the eggs, and 0.8 ml of extract mixed with a vial of 7 ml of Aquasol (New England Nuclear) and the radioactivity determined. Normal fertilized eggs at 40 min of development, whose transport rate is near maximum activa-

[1] Abbreviations used: [^{3}H]TdR, tritiated thymidine; TdR, thymidine; TMP, thymidine monophosphate; TDP, thymidine diphosphate; TTP, thymidine triphosphate; DNP, 2,4-dinitrophenol; TCA, trichloroacetic acid; PCA, perchloric acid.

tion, will take up 25% of the [^{3}H]TdR during a 5-min pulse under these conditions.

4. Washing out procaine. To remove eggs from procaine, egg suspensions were poured into 50-ml conical tubes and the eggs gently sedimented by hand centrifugation. The procaine sea water was aspirated off and the eggs resuspended in 50 ml of fresh sea water. Following two additional washes, with 5 min between washes, the eggs were resuspended in the original volume of sea water, and fresh [^{3}H]TdR added if desired.

5. Chromatography of TdR, TMP, TDP and TTP. Because transport is very low in procaine, greater isotope concentrations were used in experiments in which the composition of the TCA-soluble radioactivity in the form of TdR, TMP, TDP and TTP was determined. Two percent egg suspensions contained 4 μCi/ml of [^{3}H]TdR (6 Ci/m*M*). A 2-ml aliquot of egg suspension was washed three times in 10 ml of 0°C sea water and the final pellet approximately 0.04 ml extracted in 0.05 ml of 10% TCA. Five microliter portions of each extract were spotted on 1.5 × 20-cm strips of Whatman 3MM chromatography paper. Two microliters of a standard mix of TdR, TMP, TDP and TTP, each at 0.05 *M*, were also spotted. Thymidine and its nucleotides were separated from each other by ascending chromatography in a solvent of t-amyl alcohol:concentrated formic acid:water (3:2:1, by volume) (Suzuki and Mano, 1974; Mitra and Bernstein, 1970). The paper strips were dried and the position of TdR, TMP, TDP and TTP spots determined with a uv lamp. The strips were cut into pieces containing one spot and the pieces eluted 2 hr in 1.5 ml of 1 *N* HCl. One milliliter of eluate was mixed with 7 ml of Aquasol and the radioactivity per spot determined. As controls on percent recovery, 5 μl of each extract was spotted, dried, eluted and counted. Comparing these counts to the total counts in TdR and its nucleotides showed that 84–100% of the radioactivity could be recovered as TdR or one of its phosphorylated derivatives.

RESULTS

1. Chromosome Cycles in Procaine

The first and second cycles of chromosome condensation and decondensation of normal fertilized eggs are shown in Fig. 1. All fertilized eggs possess mitotic chromosomes by 85 min, and by 110 min decondensation is complete and the eggs are in mid-telophasic cytokinesis. During the second cell cycle, mitotic chromosomes are present in all eggs by 150 min, and they complete decondensation by 190 min when cytokinesis occurs again and the four-cell stage is reached.

Unfertilized eggs in 10 m*M* procaine begin to exhibit condensed chromatin at 90 min (Fig. 1) which is 15 min later than the fertilized eggs. Concentrations above 10 m*M* did not decrease this lag. Once initiated, the rate of condensation in procaine is equal to that of fertilized eggs. In procaine, 100% possess mitotic chromosomes by 105 min, and decondensation does not begin until after 150 min. This 45-min period of fully condensed mitotic chromosomes is more than twice the length of time mitotic chromosomes remain in fertilized eggs. Observations of individual karyotypes show the 22 maternal chromosomes to be duplicated. It is assumed that these doubled chromosomes (Mazia, 1974a) are proof that DNA replication has occurred in response to procaine treatment. In procaine, chromosome decondensation begins after 150 min and continues to 210 min. By superimposing the curve for the first complete chromosome cycle in procaine-activated eggs and the curve for the first two cycles in fertilized eggs it is evident that the chromosome cycle in procaine occurs at 50% the rate of fertilized eggs. The second cycle of condensation in procaine appears of lesser rate than the first. The reason for this discrepancy is the increasing loss, with time, of the natural synchrony among eggs. All eggs in procaine again possess mitotic chromosomes by 300 min. Observations of karyotypes at this time reveal approximately 44 doubled maternal

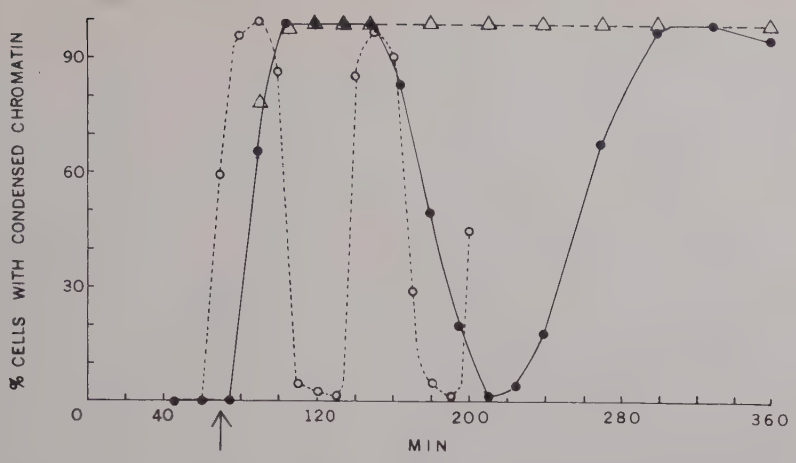

FIG. 1. Chromosome cycles of unfertilized eggs in 10 m*M* procaine (●——●) compared to fertilized eggs (○- - -○). At 75 min (arrow) procaine was removed from a portion of the unfertilized eggs and the eggs placed in sea water (△- - -△). These eggs condensed chromosomes but they did not undergo chromosome decondensation in the absence of procaine.

chromosomes per cell (Mazia, 1974a), indicating completion of the second cycle of chromosome replication.

A portion of the eggs in 10 m*M* procaine was removed at 70 min, the procaine washed out and the eggs cultured in fresh sea water. These eggs condensed chromosomes at the same time and rate as those eggs continuously exposed to procaine (Fig. 1). They did not, however, undergo chromosome decondensation and retained their set of mitotic chromosomes for the remainder of the experiment. The chromosome cycle was thus turned on by the addition of procaine, and it was turned off when procaine was removed. These eggs, fertilized 7 hr after procaine removal, developed to normal prism larvae, showing that procaine treatment had not harmed their developmental potential (Mazia, 1974a).

[^{3}H]TdR Incorporation

The incorporation of [^{3}H]TdR into DNA in the experiment shown in Fig. 1 is presented in Fig. 2. Fertilized eggs begin [^{3}H]TdR incorporation 30 min after insemination (Hinegardner, 1964). The first S-phase terminates by 45 min and the cells proceed into the first mitotic prophase. By this time 44,000 cpm are incorporated per sample. Eggs in 10 m*M* procaine possess duplicated haploid chromosomes by 105 min (Fig. 1). It is assumed, therefore, that they have completed one round of DNA synthesis. By 105 min (Fig. 2) only a small fraction (1/65) of the radioactivity is incorporated into procaine-treated eggs as compared to fertilized eggs. These results emphasize the danger of equating the incorporation of [^{3}H]TdR to the amount of DNA synthesized. It will be shown that this low incorporation in procaine results from the failure of the cells to activate an appreciable rate of [^{3}H]TdR transport before the first S-period. However, at 180 min [^{3}H]TdR incorporation rapidly rises. The time of this rise corresponds to the time of decondensation of the chromosomes and the beginning of the second S-phase (Fig. 1). Incorporation is linear from about 180 to 240 min. This is the second S-period which requires 60 min to complete. The length of the S-period in procaine is therefore four to five times longer that the S-period of fertilized eggs (Hinegardner, 1964). Incorporation levels off beginning at 240 min and continuing to 360 min. This timing corresponds to the second period of chromosome condensation in procaine (Fig. 1).

It will be remembered from Fig. 1 that a

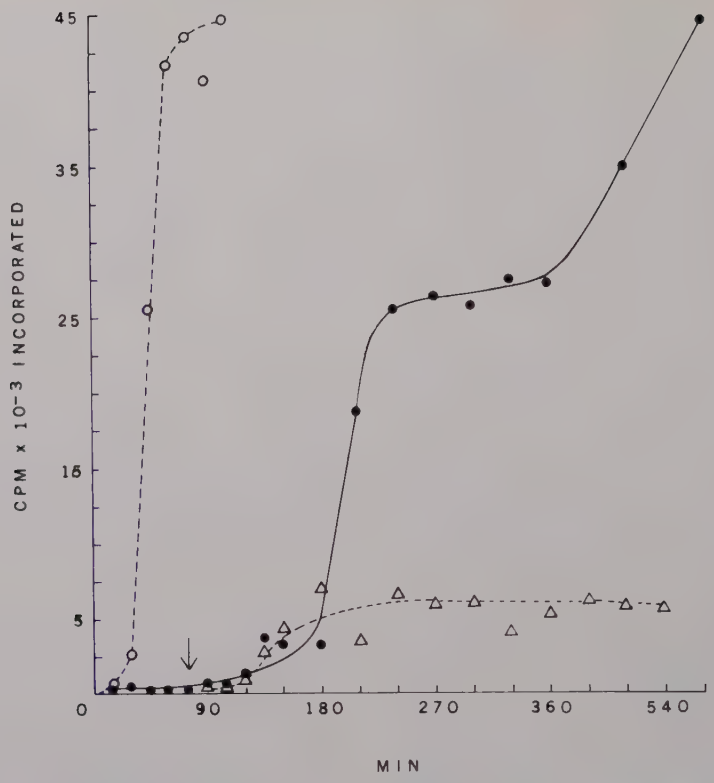

FIG. 2. Cumulative [^{3}H]TdR incorporation of unfertilized eggs in 10 m*M* procaine (●——●) compared to fertilized eggs (○- - -○); same eggs used for Fig. 1. Only the first S-phase is shown for the fertilized eggs. In procaine, very little [^{3}H]TdR is incorporated until the second S-phase beginning at 180 min. The eggs removed from procaine at 75 min (△- - -△) began the second S-phase but stopped S in the absence of procaine.

portion of the eggs in procaine was removed at 70 min (arrow, Figs. 1 and 2), the procaine washed out and the eggs returned to sea water with fresh isotope. These eggs condense chromosomes (Fig. 1) which fail to decondense for the length of the experiment. Fig. 2 shows that although these eggs begin the steep rise in [^{3}H]TdR incorporation at 180 min, they do not continue incorporation; they turn off DNA synthesis (Fig. 2) and retain their haploid set of mitotic chromosomes (Fig. 1).

3. *Turning On and Turning Off DNA Synthesis*

Because procaine-treated eggs begin chromatin condensation at 75 min (Fig. 1) it is assumed that one S-phase has occurred by this time. Because removal of procaine at 70 min does not change the rate of chromatin condensation, procaine probably has no direct effect on the mechanism of chromosome condensation. However, if procaine acts directly on the mechanism of DNA replication, removal of procaine during the steep increase of incorporation after about 180 min should turn off DNA synthesis abruptly. Unfertilized eggs were placed in 2.5 m*M* procaine and samples taken to determine [^{3}H]TdR incorporation. At 240 min, one-half the culture was carefully washed free of procaine and returned to sea water containing fresh isotope. Within 15 min of procaine removal (Fig. 3), the cells stop synthesizing DNA. Eggs continuously in procaine continue DNA synthesis. These data suggest some direct effect of procaine on the DNA synthetic machinery.

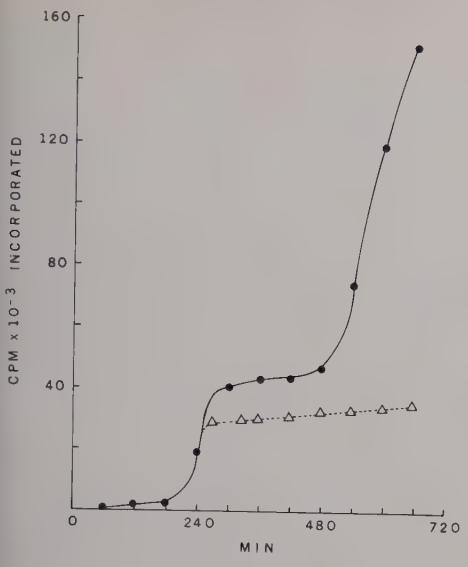

FIG. 3. Removal of procaine (2.5 m*M*) at 240 min stops further cumulative incorporation of [^{3}H]TdR into DNA of unfertilized eggs (△- - -△). Eggs remaining in procaine (●——●) show the normal pattern of DNA synthesis.

In another experiment, eggs were removed from 5 m*M* procaine at 75 min and returned to normal sea water with fresh [^{3}H]TdR. These eggs had initiated DNA synthesis (expanded-scale inset, Fig. 4) at 30 min after procaine treatment began. Within 15 min of procaine removal, they started to reduce their rate of DNA synthesis. All these eggs possessed mitotic chromosomes by 110 min (numbers adjacent to data points in Fig. 4 are percent cells with condensed chromatin) and retained these chromosomes for the duration of the experiment. A low, linear level of incorporation continues to occur in the eggs removed from procaine. This is probably caused by low level cytoplasmic conversion of the 5-methyl-^{3}H-labeled thymidine into other acid-insoluble material that is not nuclear DNA. We did not explore the reason for this low-level incorporation. At 360 min crystalline procaine was added to 5 m*M* to a portion of the eggs removed from procaine at 75 min. After a 30-min lag (Fig. 4) the chromosomes started decondensing and the incorporation of [^{3}H]TdR began to increase at a rate equal to that which occurred at 150 min in the eggs continuously in procaine. DNA synthesis can thus be turned on, turned off and turned on again by the addition, removal and re-addition of procaine.

4. *Concentration Dependency of Incorporation*

The rate of incorporation of [^{3}H]TdR into unfertilized egg DNA is proportional to procaine concentration. At concentrations above 1 m*M*, we always observe the rapid rise in incorporation during the second S-phase from about 180 to 240 min. Between these times, the rate of incorporation increased with increasing procaine concentrations up to 3.5 m*M* (Fig. 5). Above 3.5 m*M*, the amount of cumulative incorporation by 240 min remains constant. One-half the maximum rate of incorporation is obtained in 2.65 m*M* procaine. It can be argued that the observed concentration dependency of incorporation results from increases uptake of isotope or is somehow linked to the intracellular translocation and utilization of the isotope. These possible explanations are ruled out because the rates of chromosome condensation and [^{3}H]TdR incorporation are both concentration dependent. This relationship is shown in Fig. 6. Eggs were placed in 1, 2 and 4 m*M* procaine and the rates of chromatin condensation and [^{3}H]TdR incorporation determined. These data show that the concentration dependency of [^{3}H]TdR incorporation is a true estimate of the rate of DNA synthesis and chromatin condensation in response to procaine. These data show DNA synthesis in procaine to be a graded, gradual activation. The observed rate of [^{3}H]TdR incorporation is proportional to the visible rate of chromosome condensation. Procaine does not act as other parthenogenetic agents which completely throw the switch that turns on DNA synthesis at a rate equal to fertilized eggs (Steinhardt and Epel, 1974).

It can also be argued that the lack of

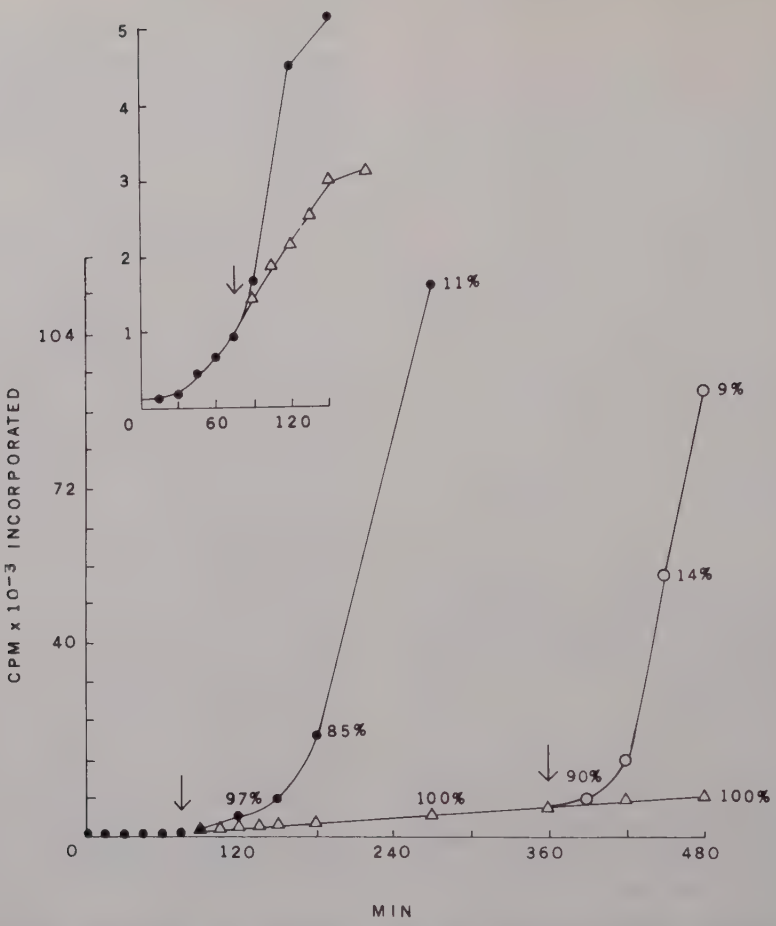

FIG. 4. Unfertilized eggs begin DNA synthesis 30 min after procaine treatment begins (5 m*M*, inset ●——●). At 75 min (first arrow), procaine was removed and the eggs began to turn off DNA synthesis (△——△). Numbers adjacent to data points give percent of cells with condensed chromatin. At 120 min the eggs in procaine began chromosome decondensation and increased DNA synthesis (●——●). The eggs removed from procaine at 75 min retained condensed chromosomes and showed only a low level of [³H]TdR incorporation. At 360 min (second arrow), crystalline procaine was added (5 m*M*) to a portion of these eggs. After a 30-min lag they began to decondense chromosomes and synthesize DNA (○——○). DNA synthesis can be turned on, turned off and turned on again by addition, removal and re-addition of procaine.

increase in rate of [³H]TdR incorporation above 3.5 m*M* (Fig. 5) results from depletion of exogenous [³H]TdR. Although isotope depletion occurs rapidly after 60 min (Fig. 7), the rate of incorporation continues to rise for many hours, showing that lack of isotope is not limiting incorporation. The curve fitting the data points in Fig. 7 shows that four periods of S occur in 12 hr. By 12 hr, fertilized eggs of this species, raised at 15°C, would have completed seven to eight cycles of S and be embryo between the 128- and 256-cell stage (Hinegardner, 1967, p. 148). Unfertilized eggs in procaine, therefore, cycle at one-half the rate of fertilized eggs. Chromosome condensation and decondensation in procaine also occur at one-half the fertilized rate (Fig. 1). Could this 50% reduction in rate reflect the fact the procaine-treated eggs contain only 50% of the amount of nuclear DNA of fertilized eggs?

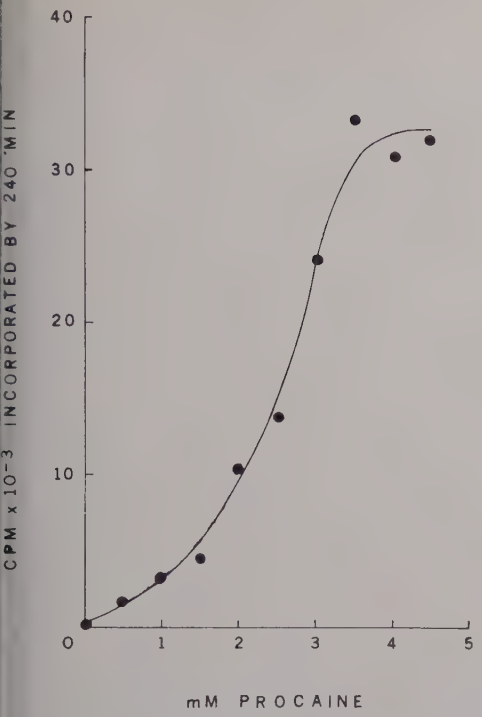

FIG. 5. Dependence of cumulative [³H]TdR incorporation on procaine concentration. Unfertilized eggs were placed in procaine from 0 to 4.5 m*M* and cumulative incorporation determined at 240 min. No increase in incorporation occurs above 3.5 m*M*. One-half the maximum rate of DNA synthesis occurs in 0.65 m*M* procaine.

. *Effect of Procaine on DNA Synthesis and Development of Fertilized Eggs*

Eggs were fertilized in normal sea water containing [³H]TdR and, at 30 min, when the first S-phase begins, the culture was divided into two lots and crystalline procaine added to one lot to a final concentration of 5 m*M*. Samples were removed during the first two S-periods and the amount of [³H]TdR incorporation determined. Procaine does not change the amount or timing of [³H]TdR incorporation for the first two S-phases in eggs which have been completely activated by fertilization. However, incorporation does decrease with time in procaine, and development arrests after several hours. For example, at 10 hr post fertilization, controls were mid-gastrulae and the 2 m*M* procaine embryos were lysed. The highest concentration of procaine at which hatching occurs is 0.5 m*M*. While control embryos hatch by 20 hr, those in 0.5 m*M* procaine begin hatching at 48 hr. The damaging effects of procaine appear to be on the membranes of blastomeres. Blebbing and extrusion of lipid droplets is common in embryos developing in procaine. Unfertilized eggs do not show signs of membrane deterioration after 12 hr in 3 m*M* procaine. The procaine concentrations used to activate DNA synthesis (2–10 m*M*) in these experiments are therefore too high to permit development of fertilized eggs.

6. *Transport of [³H]TdR Into Eggs*

The effect of procaine on transport of [³H]TdR was determined by extracting eggs with cold 5% TCA after a 5-min expo-

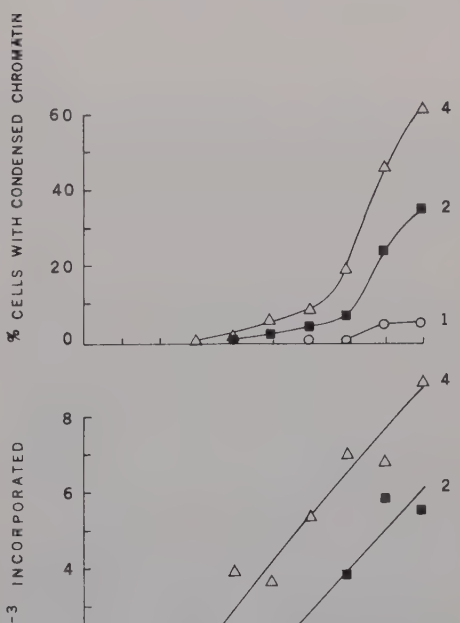

FIG. 6. Relationship between rate of [³H]TdR incorporation (bottom) and rate of chromosome condensation (top) in 1, 2 and 4 m*M* procaine. The rates of both are proportional to procaine concentration.

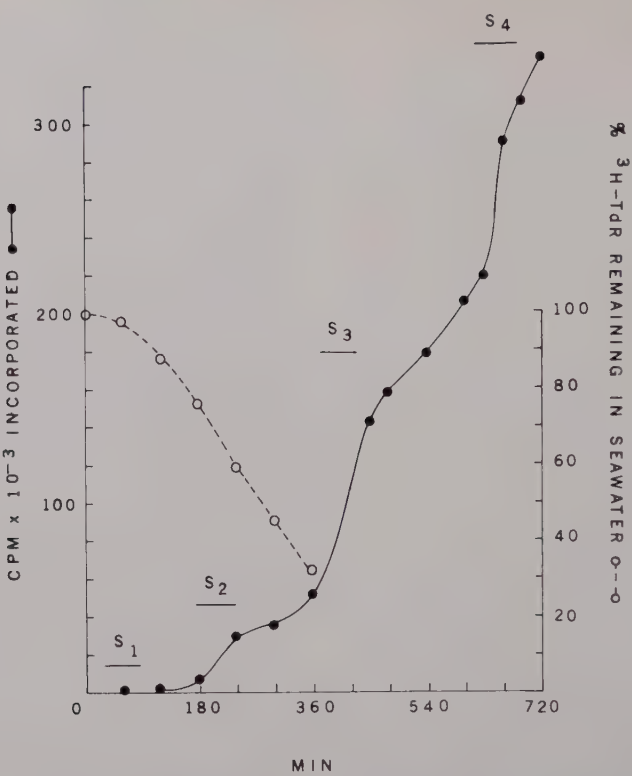

FIG. 7. Cumulative [^{3}H]TdR incorporation in procaine (4.5 m*M*, ●——●) and depletion of [^{3}H]TdR from sea water (○- - -○). Although depletion of isotope occurs rapidly after 60 min because of increased transport, incorporation increases for many hours. The steep portions of the curve represent S-phases. These procaine-treated unfertilized eggs proceed through cycles of S at 50% the fertilized rate.

sure to isotope. The activation of the transport system as a function of time was determined for procaine-treated unfertilized eggs and fertilized eggs. These data (Fig. 8) show the rapid activation of [^{3}H]TdR transport beginning at 5 min after fertilization which has been previously described by others (Piatigorsky and Whiteley, 1965; Mitchison and Cummins, 1966). As reported by these authors, the rate of activation of the transport system levels off and remains constant by 60 min. Unfertilized eggs in procaine (10 m*M*, same egg lot used for Figs. 1 and 2), however, do not begin to transport appreciable amounts of [^{3}H]TdR until 40 min. These data show that a transport mechanism is activated in procaine, but by 90 min it reaches only 2% of the rate of fertilized eggs (Table 1). In another experiment, procaine-treated eggs reached maximum rate of transport at 180 min which was only 6% of the maximum rate attained by fertilized eggs. Procaine treatment demonstrates that [^{3}H]TdR transport can be "uncoupled" from DNA synthesis (Epel *et al.*, 1974). The chromosomes condense in procaine only slightly later than in the fertilized controls (Fig. 1), which is evidence that DNA replication has occurred. The transport data for the same egg lot show, however, the transport system to be almost completely inactive.

7. *Concentration Dependence of Transport*

The activation of [^{3}H]TdR transport is also dependent on procaine concentration. Eggs were incubated in procaine ranging from 0 to 3 m*M* and samples pulsed 5 min with [^{3}H]TdR. The data (Fig. 9) show the concentration dependence of transport activation. In this experiment, maximum rate

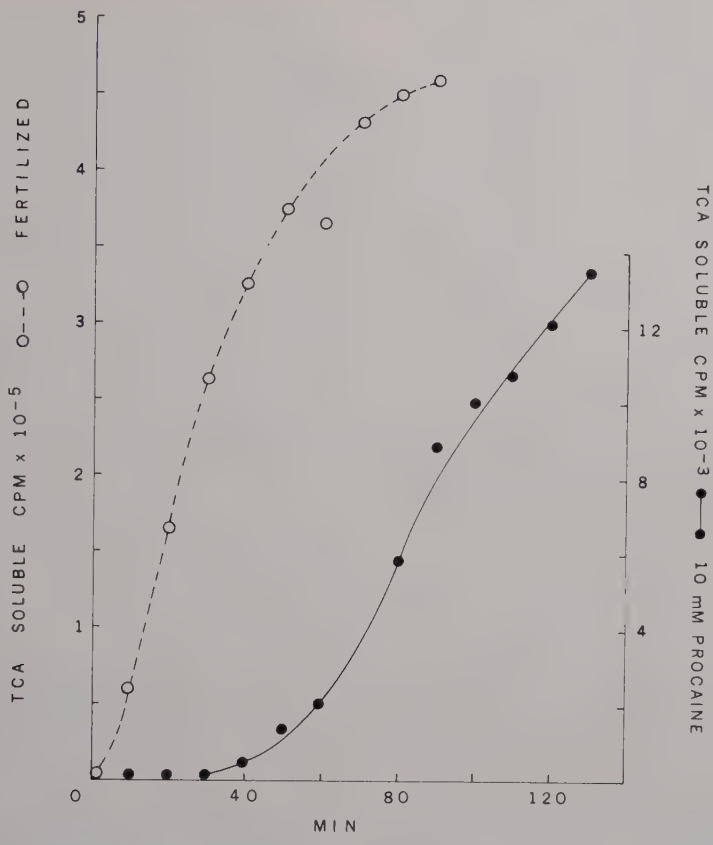

FIG. 8. Transport of [^{3}H]TdR per 5-min pulse per sample, fertilized eggs (○– – –○) compared to unfertilized eggs in 10 m*M* procaine (●——●). Note exponents on ordinates. Very little transport occurs in procaine until after 60 min (Table 1).

is reached for all concentrations by 180 min. Not only is the rate of activation of transport proportional to the procaine concentration (Figs. 9 and 10), but so is the final rate of transport achieved. This might reflect an antagonism between procaine activating transport and homeostatic mechanisms attempting to retain the egg in a metabolically inactive state. Evidence for this idea is found by noting that after 180 min (Fig. 9) a decrease in the rate of transport occurs in the lower procaine concentrations (0.6–2.0 m*M*). A plot of transport rate of 120 min against procaine concentration (Fig. 10) shows the rate to be linear from 0.5 to 1.5 m*M* with 1.15 m*M* being the concentration for one-half maximum rate of activation. It will be remembered that 2.65 m*M* procaine is the concentration to achieve half maximum rate of DNA synthesis (Fig. 5).

8. *^{3}H-TdR Transport and Procaine Removal*

The further activation of [^{3}H]TdR transport stops when procaine is removed. Eggs were placed in 5 m*M* procaine, samples pulsed for 5 min, and the amount of transport determined. At 75 min one-half the culture was washed free of procaine and returned to normal sea water and pulse labeling continued for 510 min. The data (Fig. 11) show the rate of uptake increasing from 30 to 180 min. This is consistently found between 0 and 180 min. However, the gradual decrease in transport rate occurring after 180 min in procaine was not observed in all experiments. In other ex-

TABLE 1

PERCENT RATE OF [^{3}H]TdR TRANSPORT: PROCAINE-TREATED TO FERTILIZED (%P/F)[a]

Time (min)	P/F (%)
10	0.0
20	0.0
30	0.0
40	0.2
50	0.4
60	0.6
80	1.3
90	1.9

[a] The percentage of the rate [^{3}H]TdR is transported into eggs in procaine (10 m*M*, data from Fig. 10) compared to the rate of transport in fertilized eggs for the first 90 min after beginning procaine treatment or fertilization.

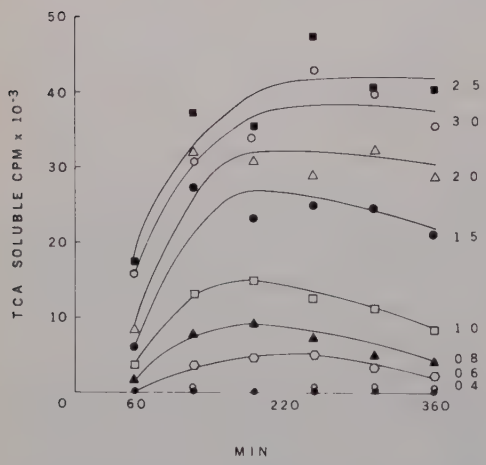

FIG. 9. Transport of [^{3}H]TdR per 5-min pulse at various times and concentrations (m*M*) of procaine. Maximum activation of the transport system occurs by 180 min.

periments the rate either levelled off at 180 min or continued to show a slight increase. The reason for this inconsistency after 180 min was not determined. Eggs removed from procaine at 75 min (first arrow) turned off further activation of the transport system. This result is explainable by proposing the idea that procaine acts by unmasking sites of transport in the cell membrane. Once unmasked, these sites remain active for several hours, losing activity at only a minor rate. The point t stress is that transport is not turned off b removing procaine as is DNA synthesis Transport continues in the absence of pro caine but only at the rate it had reached a the time procaine was removed. Re-addin 5 m*M* procaine to these eggs at 300 mi (second arrow, Fig. 11) actually causes decrease in transport rate. This is surpris ing since DNA synthesis turns on agai when procaine is re-added at 360 min (Fig 4). Both the turn-off of further activatio of transport after removing procaine an the decrease in transport when procaine i re-added later are consistently observed.

9. *Is Direct Transport across the Ce Membrane or Phosphorylation of [^{3}H TdR Responsible for the Low Rate Transport and Incorporation?*

Figure 1 shows that chromosome conde sation in procaine begins only 15 min late than in fertilized eggs, and Fig. 7 show that eggs in procaine go through cycles DNA synthesis at about 50% the rate fertilized eggs. An appreciable amount DNA synthesis therefore occurs in pro caine-treated unfertilized eggs. Howeve a very low level of [^{3}H]TdR transport an incorporation occurs in procaine (Figs. and 8). Incorporation is, of course, limite

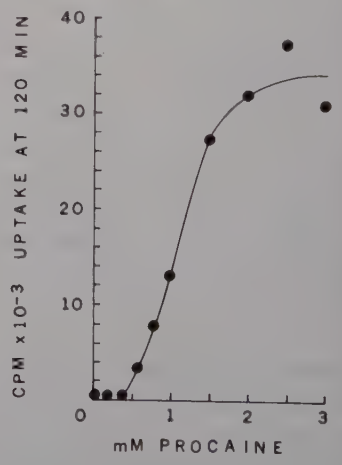

FIG. 10. Rate of [^{3}H]TdR transport at 120 min a function of procaine concentration. One-half max mum rate of activation occurs at 1.15 m*M*.

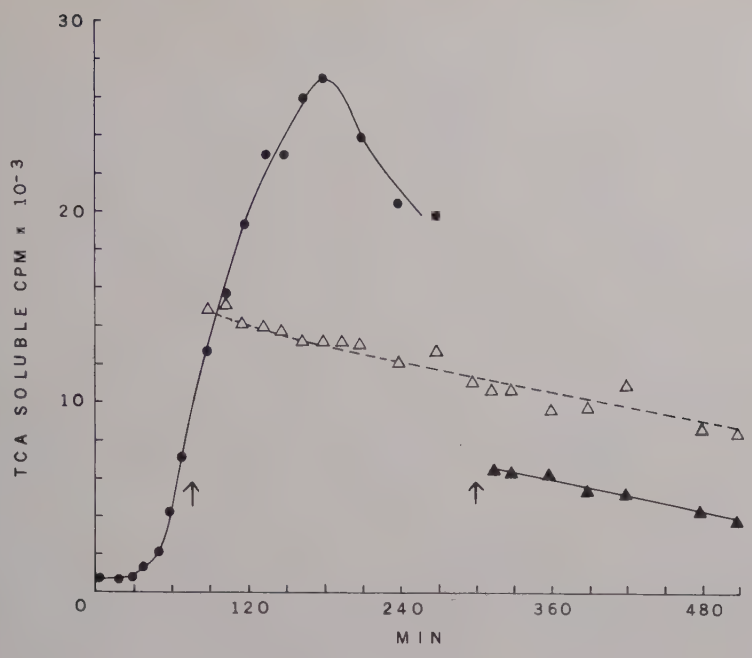

FIG. 11. Rate of activation of the [^{3}H]TdR transport system in 5 m*M* procaine (●——●). Eggs removed from procaine at 70 min do not turn off transport. The transport system remains turned on at the rate it had reached at the time procaine was removed, and only a slight decrease is observed (△- - -△). If procaine is re-added to these eggs at 300 min (▲——▲), a decrease in transport rate occurs.

by transport; therefore the question is, what could limit transport? Since nucleosides are phosphorylated after transport across the cell membrane (Piatigorsky and Whiteley, 1965; Suzuki and Mano, 1974), one can visualize a control system in which lack of phosphorylation of nucleosides inhibits direct transport of nucleosides into the cell. The deoxynucleoside kinases may not be activated in procaine-treated eggs. Evidence based on the chromatographic analysis of the acid-soluble pool of [^{3}H]TdR and its nucleotides indicates that decreased [^{3}H]TdR transport and not low phosphorylation limits [^{3}H]TdR transport and subsequent incorporation.

Eggs were placed in 5 m*M* procaine containing [^{3}H]TdR and samples taken from 30 to 390 min, extracted with TCA and the percentage of the total radioactivity as TdR, TMP, TDP and TTP determined by paper chromatography as given in the methods section. Since transport of [^{3}H]TdR is so low before 30 min, data before 30 min were not considered. The results (Fig. 12) show that at times between 60 and 390 min the intracellular TCA-soluble pool of eggs in procaine consists of 83% TTP, 13% TDP, 2% TMP and 2% TdR. The rate of total uptake is depicted by the dashed line. Thymidine kinase must indeed be active, and it will also be shown that the enzyme must be activated by procaine. The [^{3}H]TdR which enters eggs in procaine is rapidly phosphorylated to TTP. The percentage of these four compounds remains constant as the total transport increases and DNA synthesis begins again at about 180 min.

It was interesting to compare the above data from eggs in procaine to data gained from analyzing the percentage composition of the acid-soluble pool of normally fertilized eggs. Unfertilized eggs in natural sea water were incubated for 3 hr in [^{3}H]TdR and samples taken and extracted with TCA. The eggs were then fertilized

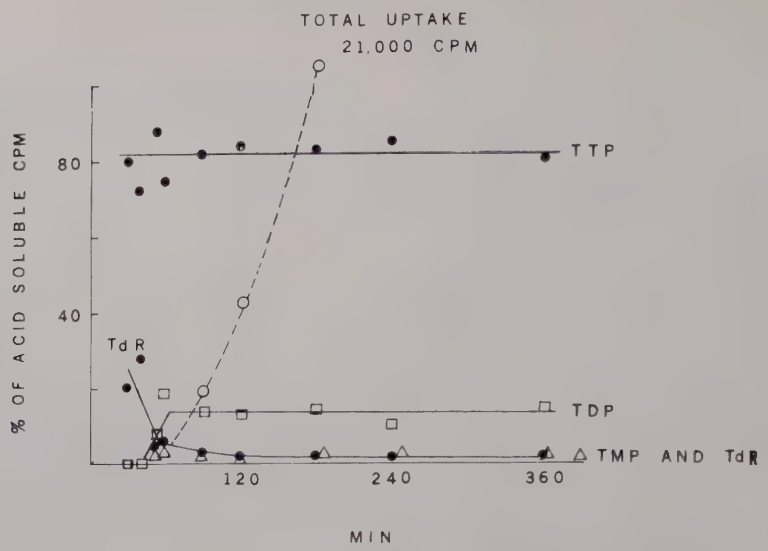

FIG. 12. Percentage composition of cumulative cold TCA-soluble extracts of unfertilized eggs in 5 m*M* procaine. Total counts per minute per 5 μl of extract (○– – –○). Only 2% of the total cpm is TdR and TMP, 13% is TDP and 83% is TTP. Phosphorylation of TdR must not be limiting its transport into the cell.

and samples taken at 5-min intervals throughout the first and second S-periods. Total uptake per 5 μl of TCA extract (Fig. 13, dashed line) increases rapidly by 5 min after fertilization (Piatigorsky and Whiteley, 1965; Mitchison and Cummins, 1966). Ninety percent of the [^{3}H]TdR permeating the eggs before fertilization remains as the free nucleoside. Thymidine kinase must not be active in unfertilized eggs, and the high level of TTP in eggs in procaine (Fig. 12) shows that procaine must activate the kinase. Figure 13 shows that in normally fertilized eggs the thymidine kinase is apparently activated at exactly 5 min after fertilization since the percentage of TTP increases sharply at 5 min. The [^{3}H]TdR transport system is relatively inactive compared to the kinase activity, so between 5 and 20 min TTP increases and a reciprocal decrease in TdR occurs. The percentage of the total radioactivity present as TTP decreases from 20 to 40 min because of its incorporation into DNA. The percentage of [^{3}H]TdR rises rapidly between 25 and 40 min. Where TdR plus TTP does not equal 100% the remaining percentage is made up by TDP. Only 1–2% of the total radioactivity can be recovered as TMP. The transport system is fully activated by 60 min, and this is the time when intracellular [^{3}H]TdR levels off at 45%, TTP at 40%, TDP at 14% and TMP at 1%. In addition to showing the activation of thymidine kinase 5 min after fertilization, these data also show that a large pool of [^{3}H]TdR exists as the free nucleoside in fertilized eggs. The percentage of TTP and TdR in fertilized eggs equals the percentage of TTP found in unfertilized eggs in procaine. It thus appears that transport of the free deoxynucleoside into eggs in procaine and not inactivity of thymidine kinase is what limits the incorporation of [^{3}H]TdR into DNA.

Data similar in principle to these, but differing in number, have been presented by other authors (Longo and Plunkett, 1973; Suzuki and Mano, 1974) working with different species. These authors plotted their data as "total counts" not as "percentage of total counts" as we have done. Our data plotted as total counts agree very closely with theirs. The percentage of TDP in both fertilized eggs and unfertilized eggs in procaine is constant at 14%. It would be interesting to use these methods

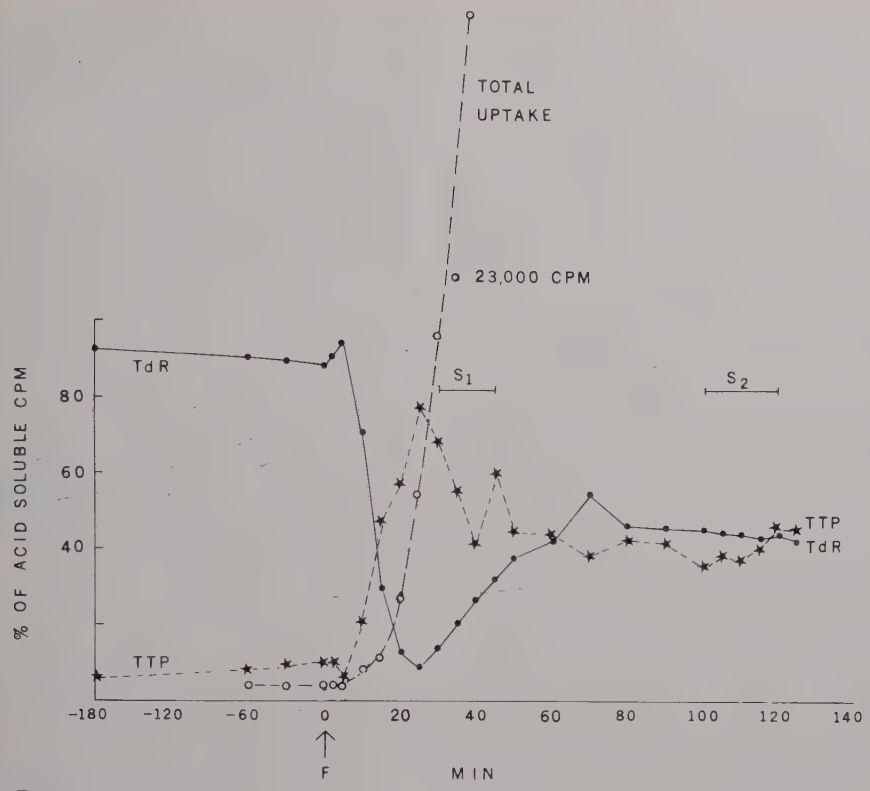

FIG. 13. Percentage composition of cumulative cold TCA-soluble extracts of fertilized eggs in the form of TdR and its nucleotides. Total counts per minute per 5 μl of extract (○– – –○). Before fertilization, what little TdR is taken up remains as TdR (●——●). At 5 min after fertilization (F), thymidine kinase is activated and TTP (★——★) increases at the expense of TdR. Percent TTP peaks at 20 min then decreases during the first S-phase (S_1) and finally levels off at 40%. Percent TdR begins to increase from 25 to 70 min when it levels off at a constant 45%. TDP increases during the initial rise in TTP and again during the rise in percent TdR after 25 min. It then becomes constant at 14% of total. TMP never exceeds more than 1% of total cpm. The high percentage of free TdR in fertilized eggs shows that direct TdR transport into the eggs in procaine and not phosphorylation is limiting uptake and incorporation in procaine.

for detailed studies of the control of the dynamics of the DNA precursor pool during the cell cycle. No deviations in the percentage composition of extracts after 80 min occurs. This is probably because the amount of transport by then is so great that differences are impossible to detect, and also the eggs are now loaded with so much exogenous isotope that only a small fraction of the total taken up is incorporated into DNA.

10. Does Procaine Treatment Release Ca^{2+} from Eggs?

The intracellular release of free Ca^{2+} is thought to be an important factor in the activation of sea urchin development (Mazia, 1937; Nakamura and Yasumasa, 1974; Steinhardt and Epel, 1974). Procaine is known to interact with phospholipids of biological membranes and cause the release of membrane-associated Ca^{2+} (Chen, 1974; reviewed by Seeman, 1972, p. 626). Could procaine activate eggs by releasing Ca^{2+}? Increasing extracellular Ca^{2+} reverses the nerve blockade produced by procaine treatment of rat phrenic nerves (Seeman *et al.*, 1974). We thought procaine-induced DNA synthesis in eggs might be turned off if external Ca^{2+} was increased. If so, this could explain the turning off of DNA synthesis after procaine removal,

natural sea water being 10 m*M* in Ca^{2+}. We found that raising the Ca^{2+} concentration to 110 m*M* at 240 min of procaine treatment does not halt DNA synthesis during the subsequent S-phase from 480 to 600 min. Thus, the only requirement for turning off DNA synthesis appears to be procaine removal.

Steinhardt and Epel (1974) loaded unfertilized eggs with $^{45}Ca^{2+}$, then treated them with the Ca^{2+} ionophore A23187. Ionophore treatment initiates fertilization membrane elevation, cortical granule exocytosis and all the biochemical activations that occur in fertilized eggs. They found $^{45}Ca^{2+}$ was released to the sea water to the same extent that occurs in fertilized eggs, and they interpret this result as the re lease of exchangeable physiological Ca^{2+} which may be directly involved in meta bolic activation. We also loaded eggs with $^{45}Ca^{2+}$, then treated them with 2 and 5 m*M* procaine and took samples of the supernat tant sea water and measured the amoun of $^{45}Ca^{2+}$ release for 7 hr. No significan release of $^{45}Ca^{2+}$ occurs (Fig. 14). We fertil ized these eggs at 7 hr and took the las sample 15 min later. The results show tha a significant release of $^{45}Ca^{2+}$ accompanie cortical granule exocytosis. It is noted tha release of $^{45}Ca^{2+}$ after fertilization of the m*M* procaine sample is low (Fig. 14). Thi results from inhibition of cortical granul exocytosis in eggs fertilized in this concen

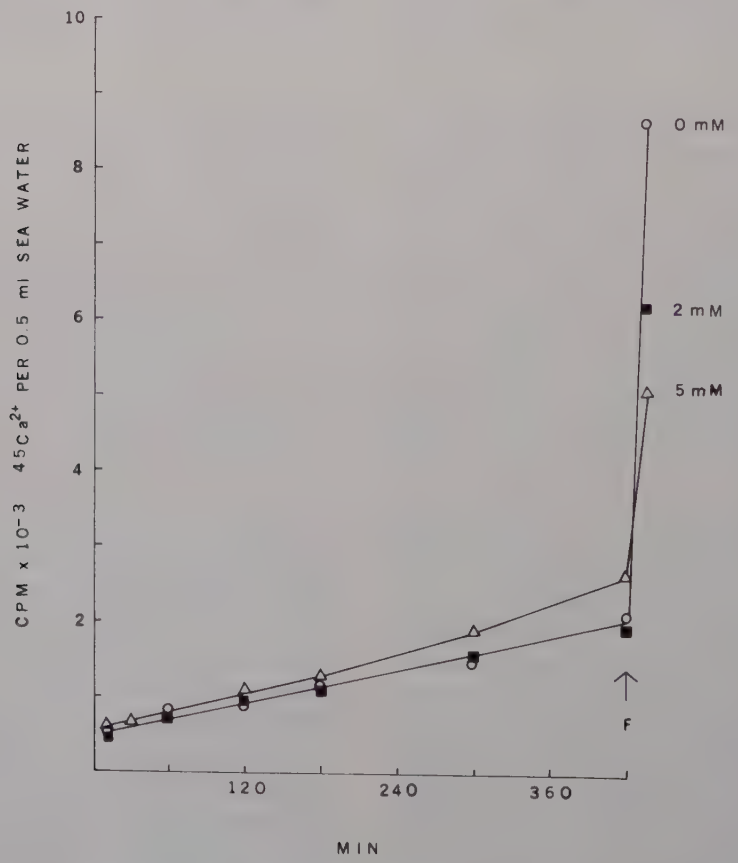

FIG. 14. Release of exchangeable $^{45}Ca^{2+}$ in procaine. Eggs were preloaded 120 min with $^{45}Ca^{2+}$, washed in sea water and placed in 0, 2 or 5 m*M* procaine. Efflux of $^{45}Ca^{2+}$ into the sea water was measured for 240 min. There is no appreciable release of $^{45}Ca^{2+}$ in procaine as compared to pure sea water. The eggs were fertilized at 420 min and the last sea water samples taken 15 min later. The exocytosis of the cortical granules causes substantial $^{45}Ca^{2+}$ release.

ation of procaine (Vacquier, 1975). We erefore conclude that procaine does not lease significant amounts of exchangea- e $^{45}Ca^{2+}$ to the external medium at con- ntrations and times of exposure we know rn on the three activations studied in is paper. This, of course, tells us nothing out what procaine may do to the intracel- lar flux of Ca^{2+}.

. *Effect of Dinitrophenol*

Eggs were placed in 5 m*M* procaine and 5×10^{-4} *M* DNP (Epel, 1972) added at 4 in. Samples were taken for 390 min and aromatin condensation, [^{3}H]TdR trans- ort and incorporation into DNA deter- ined. Absolutely no activation of these hree processes occurs in DNP. All three ust therefore depend on a product of en- gy metabolism. The finding that [^{3}H]- dR transport in procaine is energy requir- ng is in contrast to recent work supporting he view that adenine transport in sea ur- hin eggs is energy independent and occurs y simple physical diffusion (Doree and uerrier, 1974).

DISCUSSION

In this paper we show that procaine reatment of unfertilized sea urchin eggs urns on the chromosome cycle, the activa- ion of the thymidine transport system and NA synthesis. All three processes are ctivated at rates proportional to the exter- al concentration of procaine. The rates do ot increase above distinct procaine con- entrations. One-half the maximum rate f activation of the [^{3}H]TdR transport sys- em occurs at 1.15 m*M* procaine (Fig. 10) nd for DNA synthesis at 2.65 m*M* (Fig. 5). uch difference in the concentrations re- uired to achieve one-half maximum rate ay mean [^{3}H]TdR transport and DNA ynthesis are not tightly linked. After hese three processes have been turned on n response to procaine, removal of pro- aine turns them off. Activation by pro- aine is therefore reversible. Re-addition f procaine, to eggs which are turned off after procaine removal, will once again turn on DNA synthesis and the chromo- some cycle but not the further activation of [^{3}H]TdR transport. Cycles of DNA synthe- sis, as judged by chromosome cycling times (Fig. 1) and long-term [^{3}H]TdR incor- poration (Fig. 7), occur at 50% the rate of fertilized eggs. [^{3}H]TdR incorporation into DNA, however, is only a small fraction of that occurring in fertilized eggs. This low incorporation results from the inactivity of the [^{3}H]TdR transport system and not from lack of phosphorylation after the nucleo- side becomes intracellular. The transport system is much more active after 180 min of procaine treatment, and during the sec- ond S-phase (from about 180 to 240 min) an appreciable amount of [^{3}H]TdR incorpora- tion into DNA is observed (Figs. 2 and 3).

Ammonia treatment of unfertilized sea urchin eggs induces these same activa- tions (Loeb, 1913; Mazia, 1974a; Mazia and Ruby, 1974; Epel *et al.*, 1974). To achieve activation, eggs are treated with 1 m*M* NH_4OH in sea water for 60 min and then returned to natural sea water. Chromo- some cycles, [^{3}H]TdR transport and DNA synthesis all stay turned on in the absence of ammonia. Procaine may act closer to the ultimate controls of these activations than does ammonia because, unlike ammonia, procaine activation is sustained only in the presence of procaine. The lowered rate of [^{3}H]TdR transport, which increases with time, is also found in ammonia-treated eggs (Mazia and Ruby, 1974). Eggs acti- vated with ammonia do not form mitotic spindles and cytokinesis does not occur (Mazia, 1974a). Large cytasters (Dirksen, 1961) may develop in procaine-treated eggs (2–5 m*M*) and the eggs may cleave. The percentage cleaving varies greatly. Ide- ally, eggs in 4.5 m*M* procaine at 15°C will begin to cleave at between 210 and 360 min. In procaine, astral rays appear to emanate from what would be late-prometa- phase nuclei and electron micrographs show the rays to contain microtubules. Both astral-ray formation and cytokinesis

are enhanced when eggs, which have been in procaine for 240 min at 15°C, are warmed to 23°C. This temperature change would favor the formation of microtubules.

Interesting parallels exist between this study of [^{3}H]TdR transport and incorporation into DNA and a similar study of amino acid transport and incorporation in ammonia-treated eggs (Epel *et al.*, 1974). Amino acid transport does not occur for 45 min in ammonia, but protein synthesis turns on at 5 min and at a rate dependent on the concentration of ammonia. Similarly, we also find the rates of chromosome condensation, [^{3}H]TdR transport and DNA synthesis to depend on procaine concentration. We also find that appreciable [^{3}H]TdR transport does not occur until about 45 min. These data and those of Mazia and Ruby (1974) show that [^{3}H]TdR transport can be uncoupled from DNA synthesis (Epel *et al.*, 1974). Tupper (1973) and Epel *et al.*, (1974) have shown that protein synthesis continues after amino acid transport has been artificially blocked. Macromolecular synthesis in these cells may therefore not be causally linked to the transport of precursor molecules (Epel *et al.*, 1974).

Unfertilized eggs in ammonia develop increased K^+ conductance which lowers the cell membrane potential from -10 to -70 mV (Steinhardt and Mazia, 1972). Elevating the external K^+ to 158 m*M* brings the membrane potential back to -10 mV. Orr *et al.*, (1972) found DNA synthesis in BHK cells can be reversibly inhibited by increasing K^+ and they related this effect to K^+ conductance at the cell membrane. We wondered if raising the K^+ content to 158 m*M* would stop procaine-activated DNA synthesis. Eggs were incubated in 2.5 m*M* procaine and at 210 min a portion of the culture made 158 m*M* in KCl. We found that DNA synthesis did not turn off in high external K^+. K^+ conductance across the cell membrane may not be a direct controller of DNA synthesis (Tupper, 1973).

Procaine may activate eggs by causing expansion and increased fluidity of the cell membrane, perturbations characteristic of local anesthetics (Seeman, 1972, p. 615; Sheetz and Singer, 1974). We know the egg cell membrane greatly expands at fertilization as a result of the fusion of cortical granule membranes with the egg cell membrane (Vacquier, 1975). The egg does not change its overall diameter after fertilization and the expanded surface is projected as numerous microvilli (Endo, 1961). Electron micrographs of unfertilized eggs activated by procaine show the villi to be longer than those of control eggs. An expansion of the cell membrane by intercalation of procaine could be the underlying mechanism of this artificial activation. Membrane expansion may also explain the apparent "unmasking" of [^{3}H]TdR transport sites which remain functional after procaine removal.

Regarding DNA synthesis, three lines of evidence indicate the procaine effect to be on some component of the DNA synthetic machinery and not on the mechanism of chromosome condensation. First, removal of procaine at 70 min (Fig. 1) does not stop chromosome condensation. Second, removal of procaine during active DNA synthesis (Figs. 2 and 3) stops the second S-period abruptly. Third, re-adding procaine to eggs with condensed chromosomes initiates decondensation and DNA synthesis (Fig. 4). One may ask if the mechanism of procaine activation might be directly on the initiation of chromosome decondensation. In sperm pronuclei we see the decondensation of chromatin occurring in the 30-min period between sperm entry and pronuclear fusion (Longo and Anderson, 1968; Longo and Plunkett, 1973). Condensed chromatin is not seen in the female pronucleus which is therefore referred to as an interphase nucleus. The chromatin, although not visually appearing condensed by cytological observation, may be biochemically as condensed as that of the male pronucleus in regard to the ability of both pronuclei to be templates for DNA

eplication. The 30-min lag between insemination and initiation of S occurs both in ertilized eggs (Hinegardner *et al.*, 1964) nd procaine-treated eggs (expanded-scale nset, Fig. 4). Could chromatin decondensaion in both male and female pronuclei be ne of the direct steps initiating DNA synhesis? The 30-min lag after re-addition of rocaine, before renewed DNA synthesis n turned-off eggs with mitotic chromoomes (Fig. 4), could implicate a decondenation mechanism to explain procaine-induced DNA synthesis. Procaine could act ndirectly by neutralizing some factor necessary for chromosome condensation and hus allow decondensation and hence DNA replication to begin. Johnson and Rao (1970) have shown that interphase cells condense chromosomes ahead of schedule if fused with cells containing condensed chromosomes, which is proof for the existence of condensation factors. The factors controlling the initiation of S may be decondensation factors, since fusion of an S-phase HeLa cell with a G_2 cell causes decondensation of G_2 chromatin (Rao *et al.*, 1975). The same factor may control chromatin decondensation and DNA synthesis.

We often speak of a cell making a "decision" which sets it on a replicative pathway (Mazia, 1974b). In normal eukaryotic cells which possess a distinct G_1-phase, the cells must synthesize RNA and protein to initiate and sustain DNA replication (Seki and Mueller, 1975). Sea urchin eggs are a different kind of cell in that the decision to replicate has been made in the ovary. They possess abundant stores of DNA polymerase (reviewed by Loeb *et al.*, 1971; Fansler, 1974) and contain the highest concentration of deoxynucleotide triphosphates measured to date in any cell (Mathews, 1975). They possess a large pool of ATP (Epel, 1969) and contain high levels of deoxynucleotide kinases (Suzuki and Mano, 1974). Inhibitor studies show protein synthesis is not required for at least the first S-phase (Black *et al.*, 1967; Epel *et al.*, 1974). These cells can be literally thought of as machines for making DNA which possess no G_1 and only a short G_2-period (25 min) and essentially cycle from S to M to S. If a G_1-phase exists it is the 30-min period between fertilization and the beginning of [^{3}H]TdR incorporation. The same time-lag is found in procaine-treated eggs. It is a wonder that cells, primed for DNA synthesis as these are, can hold themselves back from active synthesis. Could an active homeostatic mechanism keep synthesis turned off? Until this study, all agents that triggered DNA synthesis did so in an irreversible manner. The fact that DNA synthesis and the chromosome cycle turn off after procaine removal tells us the cell can reestablish the constraints that keep these replicative processes turned off. It is interesting to mention here that we cannot, as yet, get protein synthesis to turn off when procaine is removed. Procaine will prove to be a useful tool for further studies of the control of DNA synthesis and the activation of development.

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Note added in proof. Gourlie and Infante, Biochem. Biophys. Res. Commun. **64**, 1206–1214 (1975), report similar fluctuations in TTP levels before and during the first S-phase in eggs of *S. purpuratus*.

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Ultrastructural Evidence of Contractile Systems in Mouse Palates prior to Rotation

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Previous studies have shown that the palatal shelves of mouse embryos synthesize the contractile proteins actin and myosin at a rate equal to that of tongue just prior to shelf movement (day 14.5). The purpose of this study was to examine the morphology of the palatal shelves for evidence of a contractile system. Myosin ATPase histochemistry was performed on frozen sections of day-14.5 fetal mouse heads. Three areas of the palatal shelves gave a positive reaction: 1) A reaction product typical of skeletal muscle on the oral side of the posterior palate (region 1); 2) a "heavy-diffuse" reaction product on the tongue side extending from the top mid-palate to the posterior end (region 2); and 3) a "light-diffuse" reaction product along the oral epithelium in the mid-palate (region 3). Electron microscopy of excised day-14.5 palates was carried out after fixation in glutaraldehyde or an acrolein–dichromate solution. Region 1 contained a large area of developing and adultlike skeletal muscle. In the area of region 2 a large population of filamentous-rich mesenchymal cells was observed. In addition, large neurons coursing through both contractile systems were noted. Preliminary observations in region 3 indicated the possibility of a primitive (nonmuscle) contractile system in that area. The contractile and nervous systems in the palate, prior to rotation, indicate the possibility that an innervated embryonic muscle system may provide the "intrinsic shelf force" to rotate the shelves.

INTRODUCTION

Between day 14.5 and day 15.5 of gestation, the palatal shelves of fetal mice rotate from a position lateral to the tongue to a horizontal position above the tongue and shortly thereafter fuse to complete palate formation (Walker and Fraser, 1956). Peter (1924) and Lazarro (1940) proposed that the vertical palate shelf achieves its horizontal position by undergoing medial rotation in a "barndoor" fashion. However, Polzl (1904) and Pons-Tortella (1937) held the view that the horizontalization of the shelf takes place by a "remodeling" of the shelf. Further support of this comes from work of Wragg *et al.* (1972) concerning the spatial relationships within the oral cavity at the time of shelf rotation. Coleman (1965) observed that the posterior portion of the palate shelf was in a vertical position while the anterior portion was ventromedial before movement and concluded that the posterior shelf moves by remodeling while the anterior end moves by the barndoor swinging of the shelf to the horizontal position.

In recent years it has been suggested that morphogenetic movements may be due to the presence of intracellular microfilaments composed of actin polymers (see Wessels *et al.*, 1971). Furthermore, evidence is accumulating that myosin plays a role in contractile processes involving microfilaments and other primitive contractile systems, such as found in fibroblast spreading, ameboid cytoplasmic streaming, and platelet contraction (Pollard and Weihing, 1974). Investigations carried out at the light microscopic level have not re-

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ealed the existence of any contractile sys-
m within the palate shelf prior to rota-
on. No systematic ultrastructural studies
ave been done on the palate at this time
f development, although the fine struc-
ural changes in the medial edges of
helves associated with fusion have been
ell studied (Hayward, 1973; Waterman *et*
l., 1973). Thus the possibility exists that a
ystem of developing skeletal or smooth
uscle, or some primitive contractile appa-
atus not resolved by light microscopy, is
resent in the palate.

Glucocorticoids are known to produce
left palate in mice (Walker and Fraser,
957; Zimmerman *et al.*, 1970). Not only is
riamcinolone the most potent glucocorti-
oid in producing cleft palate in mice
Walker, 1965) but it also exerts the great-
st myopathic effect in humans (Jenkins,
961) and mice (Faludi *et al.*, 1966). It has
een postulated that triamcinolone exerts
s teratogenic effect (cleft palate) by a gen-
ralized embryomyopathy that is responsi-
le for decreased embryonic movements in
he muscle of the neck and mouth regions
Walker, 1971). Alternatively, it is possi-
le that the teratogenic effect may be due
o a myopathic effect on contractile ele-
ents residing in the palate itself.

Much controversy has been centered on
hether the force necessary to move the
helves actually resides inside this tissue
intrinsic shelf force") or whether the
rce develops from the outside of the shelf.
possible candidate for an intrinsic shelf
rce would be the molecular interactions
f the contractile proteins actin and
yosin. However, a variety of other mech-
nisms have been offered to account for
alate shelf rotation: Mucopolysaccharide
nthesis in the palate (Larsson, 1962),
hich has been disputed (Nanda, 1970; An-
ew and Zimmerman, 1971); nonpalatal
uscular contractions (Walker, 1969); and
raightening of the cranial base (Verru-
o, 1970). Nevertheless, no real under-
anding of this phenomenon has been ob-
ined.

Our initial experiments dealing with this problem revealed that the palate, just prior to shelf rotation (day 14.5), synthesized actin and myosin at a rate equal to that of the tongue, with the sum representing as much as 10% of the total protein synthesized (Lessard *et al.*, 1974). Therefore, ultrastructural studies have been carried out to search for the putative contractile system in the palate shelves. The studies reported here provide morphological evidence for both skeletal muscle and microfilamentouslike masses in the posterior region of the palate. Large nerve bundles are also associated with these contractile systems. A preliminary report of these studies has been presented (Zimmerman *et al.*, 1973).

MATERIALS AND METHODS

A/J mice (Jackson Labs) were used in these studies. Mating was carried out by placing individual females with males. The presence of a vaginal plug the following morning was taken as evidence of pregnancy and was designated day 0.5. Pregnant animals were killed by cervical dislocation. The gravid uteri were removed immediately and placed on ice. The fetuses were delivered and the fetal heads were removed for further processing.

Myosin ATPase histochemistry. Whole heads removed from day-14.5 fetuses were immediately frozen in liquid nitrogen and sectioned at 7–10 μm on a cryostat. The sections were picked up on subbed slides and incubated and stained for myosin ATPase following the technique of Padykula and Herman (1955). Alternate slides were stained with hematoxylin and eosin. The sections were mounted under #1 coverslips with glycerine jelly and viewed and photographed with a Zeiss photomicroscope using bright field and Nomarski interference optics.

Electron microscopy. Whole heads of day-14.5 fetuses were fixed in 2% glutaraldehyde in 0.1 *M* cacodylate buffer (pH 7.3) for 1 hr at room temperature and then

transferred to 0.1 *M* cacodylate buffer (pH 7.3) containing 5% sucrose for dissection. Whole palates were removed with fine forceps and dissected with finely drawn tungsten needles into four approximately equal pieces: 1) Top anterior palate, 2) bottom anterior palate, 3) top posterior palate, and 4) bottom posterior palate. The excised whole palates or dissected quadrants were then postfixed in Millonig's osmium (Millonig, 1962) for 1 hr, rinsed in distilled water and dehydrated in a graded series of ethanol. After three changes of propylene oxide, the tissues were infiltrated and embedded in pure Epon (Luft, 1961). Silver and gold sections were cut with a Dupont diamond knife on Porter–Blum MT-2 or Reichert OMU-3 ultramicrotomes, collected on naked 300-mesh grids, and stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963). The material was viewed and photographed on Hitachi HU-125E and Zeiss EM-9S electron microscopes. Corresponding 1-μm sections were cut and examined either unstained or stained (Richardson *et al.*, 1960) with phase contrast and bright field optics, respectively.

To preserve the ultrastructure of filamentous systems in the palate, whole fetal heads were incubated in hyperosmotic Ringer's solution for 12 hr (Small and Squire, 1972) and fixed in an acrolein–dichromate solution (Robinson and Lipton, 1969). After postfixation in Millonig's osmium, the palates were dissected, washed, dehydrated, and processed as above.

RESULTS

In an attempt to localize a contractile apparatus in the palate shelf prior to shelf rotation, myosin ATPase histochemistry was performed on whole frozen heads of fetal mice at day 14.5 of gestation. The deposition of the positive reaction product for myosin ATPase was mainly limited to two regions in the posterior half of the palate. On the oral side of the posterior end of the palate (region 1 of Fig. 1), a reaction product was observed that was indistinguishable from the reaction product of the developing myofibrils of the tongue. In the top portion of the posterior palate (region 2 of Fig. 1), a "heavy-diffuse" reaction was found that continued forward in serial sections approximately halfway to the anterior limit of the palate (Fig. 2). Within the heavy-diffuse area (region 2) occasional well-defined structures were delineated by the positive reaction product for myosin ATPase (Fig. 3). Such structures seemed to indicate the presence of a potentially well-defined contractile apparatus in this area. More anteriorly in the palatal shelf, a "light-diffuse" reaction product for myosin ATPase was noted in the mesenchyme just beneath the oral epithelium (Fig. 4). In this section a capillary is seen which also produced a positive reaction due to the presence of smooth muscle myofilaments in the capillary endothelial cells.

To examine the morphology of the palate shelf, a cross-sectional survey through the shelf was done by use of both light and electron microscopy. Thick sections of the posterior palate, examined by light microscopy, confirmed the presence of skeletal muscle in region 1 (Fig. 5, inset). Within this region, fingerlike projections of skeletal muscle were found penetrating up to 40% deep into the palate shelf (Fig. 5). These developing myotubes were often surrounded by presumptive myogenic cells (Fig. 5). There appeared to be a diverse orientation of the skeletal muscle fibers in the region. Both longitudinally (Fig. 6a) and cross-sectionally (Fig. 6b) cut myofibrils were observed in a single thin section. Numerous large nerve bundles were also observed within region 1 (see Fig. 5, inset). However, definitive myoneural junctions have not yet been positively identified. The wide spectrum of stages of skeletal muscle development observed indicated that region 1 was developing into a large skeletal muscle mass, most likely the palatopharyngeal muscle of the adult soft palate (see Morley, 1970).

For direct analysis of the ultrastructura

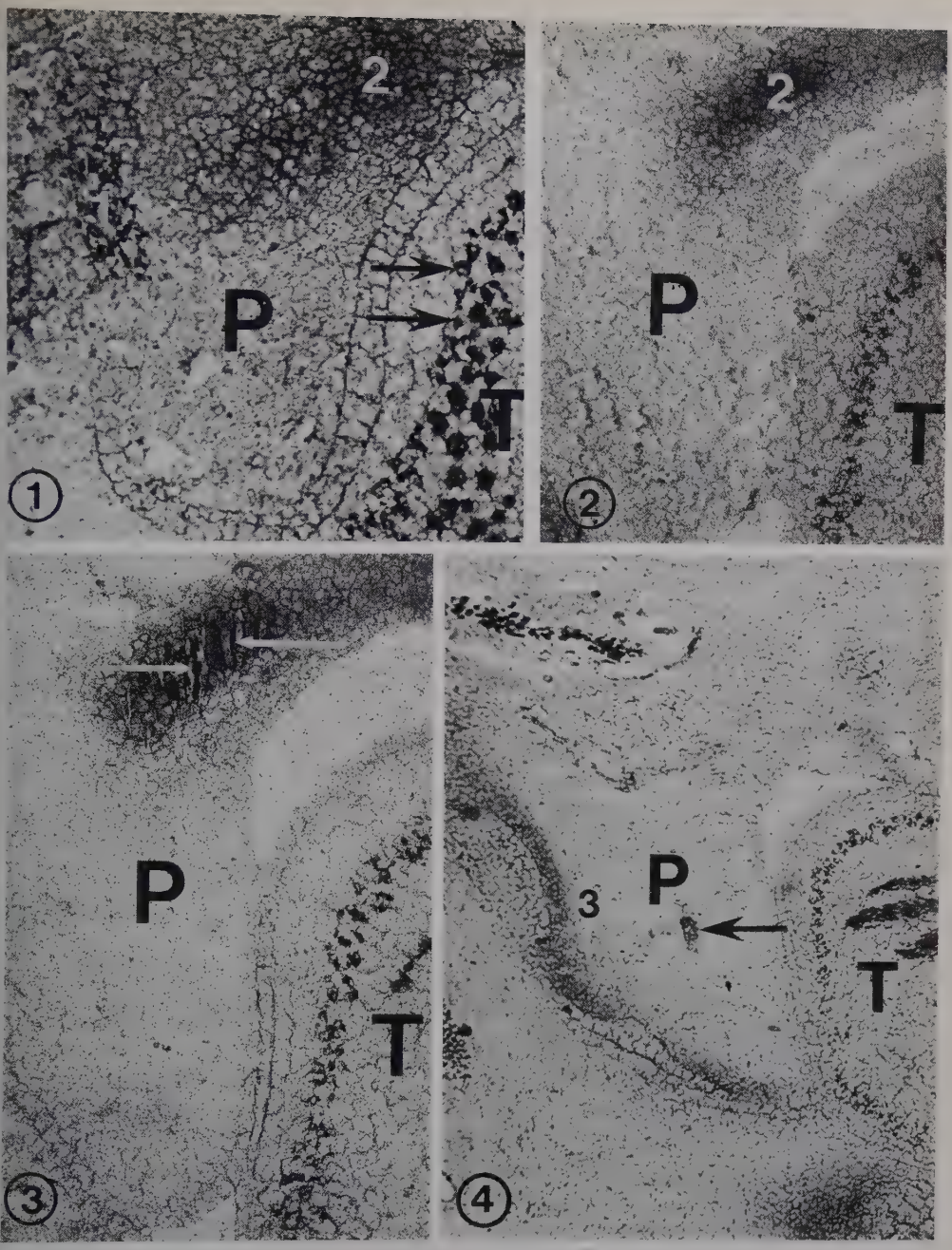
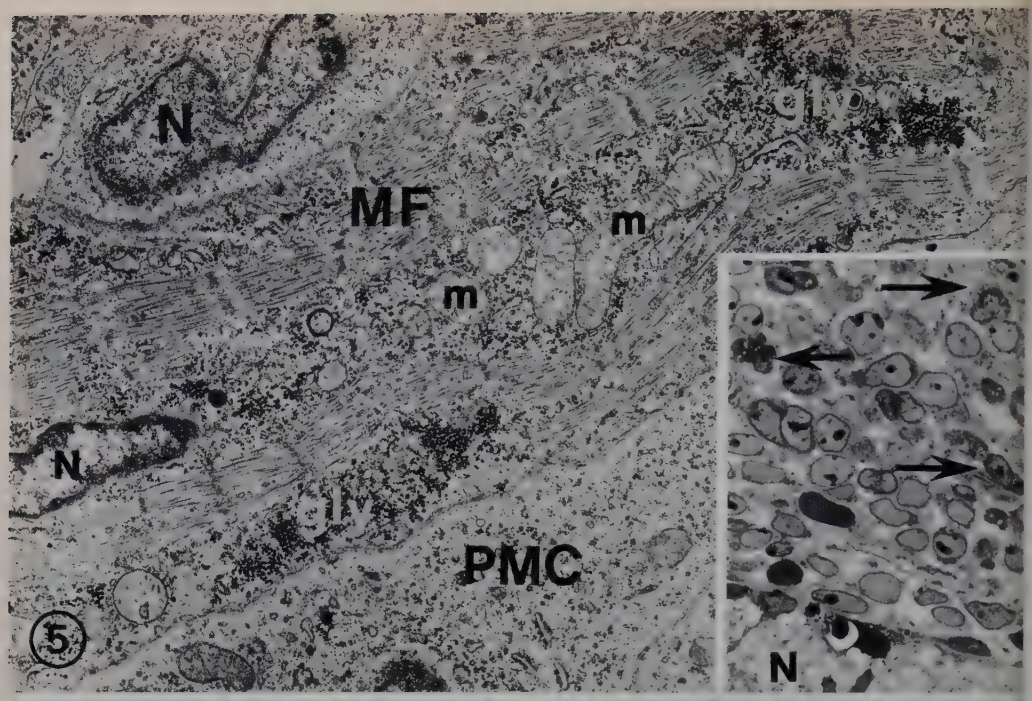


FIG. 1. A cryostat section through a day-14.5 fetal mouse head showing the positive reaction product for myosin ATPase in the posterior palate shelf (P). Region 1, on the oral side of the shelf, shows a reaction product similar to that of the developing myofibrils (arrows) of the tongue (T). Region 2, in the top posterior shelf, exhibits a heavy-diffuse reaction product which is visible over a large population of cells. × 360.

FIG. 2. A frontal cryostat section through the mid-palate (P) showing the anterior limit of the heavy-diffuse reaction product of region 2. T, tongue. × 270.

FIG. 3. An example of organized structures (arrows) found within the myosin ATPase reaction product of region 2 of the palate (P). T, tongue. × 300.

FIG. 4. A cryostat section through the anterior half of the palate (P). A light-diffuse myosin ATPase reaction product is visible in the mesenchyme immediately beneath the oral epithelium (region 3). In the center of the shelf, a positive reaction is seen associated with the smooth musculature of a capillary (arrow). T, tongue. × 150.



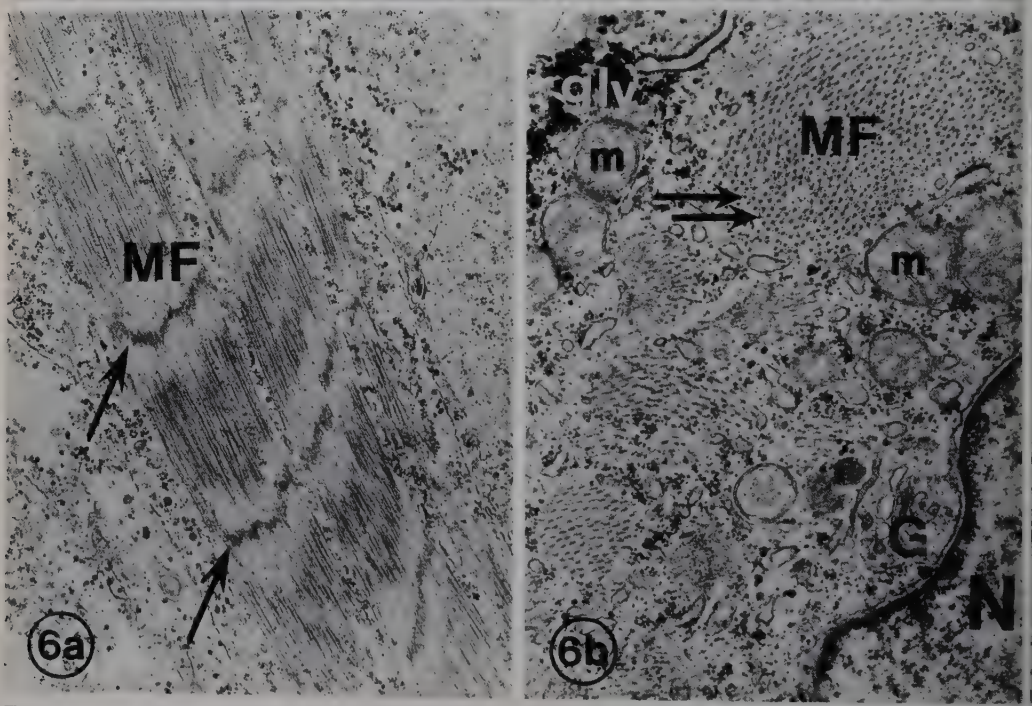


FIG. 5. An electron micrograph of a skeletal muscle myotube, containing well-developed myofibril (MF), found penetrating deeply (approximately 40%) into the palate shelf. A presumptive myogenic cel (PMC) is seen in close association with the myotube. gly, Glycogen; m, mitochondria; N, nuclei. × 9300 Inset: A light micrograph of a thick (1.0 μm) Epon section of the posterior palate shelf. A large population o obliquely cut skeletal muscle cells (arrows) and a large nerve ganglion (N) are seen. × 360.

FIG. 6. Electron micrographs of diversely oriented skeletal muscle myofibrils found in a single thi section of the posterior palate shelf. (a), A longitudinally sectioned skeletal muscle myofibril (MF) of regio 1 showing organized sarcomeric units characterized by the alignment of z-band materials (arrows). × 20,700. (b), A transversely sectioned myofibril (MF) found in the same section. The thicker (130 Å) myosi filaments (arrows) and associated thin (55 Å) actin filaments are observed. G, golgi complex; gly, glycogen m, mitochondria; N, nucleus. × 31,000.

characteristics of the area corresponding to the myosin ATPase reaction in region 2, pieces of the top posterior palate were examined with both light and electron microscopy. Thick sections of the top posterior palate showed several large nerve ganglia sending numerous postganglionic fibers into the palate shelf, including the myosin ATPase positive cell mass of region 2 (Fig. 7). Ultrastructurally, after routine fixation with glutaraldehyde and osmium tetroxide, large bundles of unmyelinated axonal processes, 680 nm (500–800 nm) in diameter, containing both small, 100-Å filaments and 250-Å microtubules, were clearly visible (Fig. 8). Likewise, after hyperosmotic incubation and fixation in an acrolein–dichromate solution, the same ultrastructural characteristics of the axonal processes were preserved (Fig. 9). Light microscopy also revealed that cellular projections of the cells of region 2 were packed together forming large bundles (Fig. 10a). Ultrastructurally, these projections were notably thinner in diameter (220–300 nm) than axonal processes and appeared densely packed with amorphous material (Figs. 10b and c). At times, within the amorphous matrix of these projections, small filaments, 70 Å in diameter, were resolved giving the impression that these projections were densely packed with randomly oriented filaments. In Fig. 9, a large bundle of filaments of the same size (70 Å) were seen within the cell body of a cell in close proximity to an unmyelinated nerve bundle. Many of the mesenchymal cells of region 2 possessed a great amount of filamentous material. Primarily, small, 70-Å filaments, with occasional thick (130 Å) filaments, were found aligned parallel to the plasma membrane and densely packed within cellular projections (Fig. 11). The interdigitating plasma membranes of the filamentous-rich cells were connected by specialized junctions, primarily fasciae adhaerentes (Fawcett and McNutt, 1969) which are characterized by electron-dense plaques on adjacent membranes and a 150–200-Å gap between membranes (Fig. 11). As in region 1, numerous nerve processes were found coursing through region 2. However, nerve terminals associated with the plasma membranes of the filament-rich cells again have not been positively identified. In summary, the cells of region 2 contained large amounts of small (70 Å) intracellular filaments packed into elongated cellular projections and running parallel to the plasma membrane.

The amorphous appearance of the components of the fibrillar systems in the palatal cells of region 2 was most likely the consequence of poor preservation of these filaments by the fixatives normally used in electron microscopy. To improve the preservation of the filamentous systems in region 2, incubation of the mouse head in hyperosmotic Ringer's solution and acrolein–dichromate fixation was performed by the method of Small and Squire (1972). The technique accentuated the presence of numerous 100-Å filaments within the cells of region 2 but had little effect on the filamentous systems observed in the elongated cellular projections (Fig. 12). The 100-Å filaments were not only localized in region 2 but were also found in region 1, which contained the skeletal muscle (Fig. 13). The 100-Å filaments are not clearly discernable in this micrograph, due in part, to the normal glutaraldehyde fixation procedure employed. These filaments were found either as distinctly organized structures (Fig. 12) or as randomly oriented filaments winding throughout the cytoplasm (Fig. 13). A contractile nature for these filaments can only be implied, but it is possible that these filaments are responsible for the light-diffuse reaction of myosin ATPase observed in region 3 (Fig. 4).

DISCUSSION

A three-dimensional drawing that summarizes the observations made of the presumed contractile systems observed in the

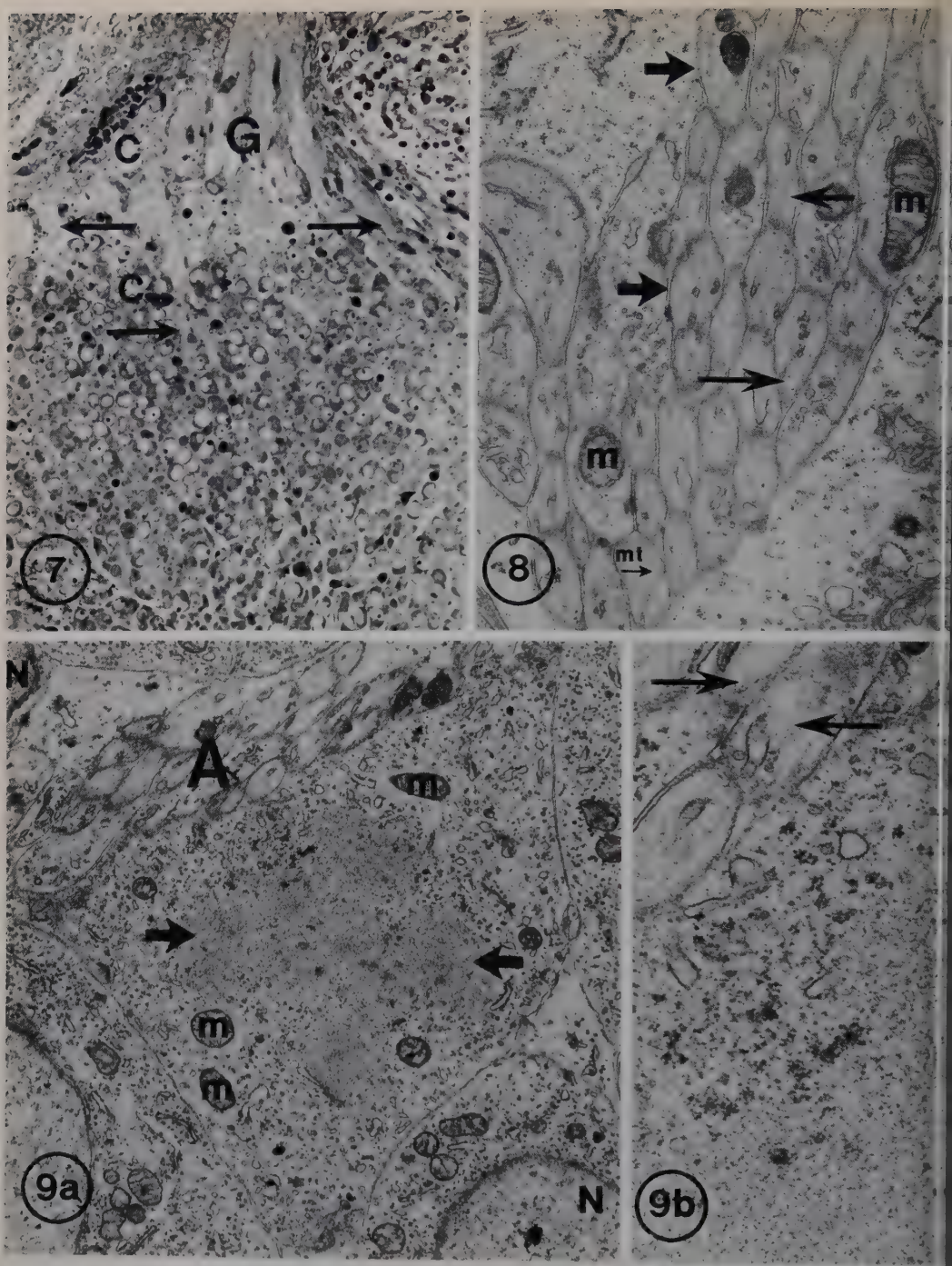


FIG. 7. A light micrograph of a thick (1.0 μm) Epon cross-section through the top posterior palate. A large nerve ganglion (G) is observed sending numerous postganglionic fibers (arrows) into the cell mass of region 2. c, Capillaries. × 495.

FIG. 8. An electron micrograph of a bundle of unmyelinated nerve processes coursing through region 2 of the palate shelf, after routine fixation with glutaraldehyde and asmium tetroxide. Small (averaging 600 nm in diameter) individual axonal processes (large arrows) containing 100-Å neurofilaments (small arrows) and 250-Å microtubules (mt-arrow) are seen. m, Mitochondria. × 126,000.

FIG. 9. (a), An electron micrograph from region 2 following hyperosmotic incubation and acrolein fixation. A small bundle of axons (A) are seen in close association to a cell containing a large bundle of 70-Å filaments (large arrows) cut in cross-section. m, Mitochondria; N, nuclei. × 7800. (b), An electron micrograph at higher magnification showing the scattered 100-Å neurofilaments (arrows) present in the nerve processes and the 70-Å filaments within the adjacent cell. × 25,000.

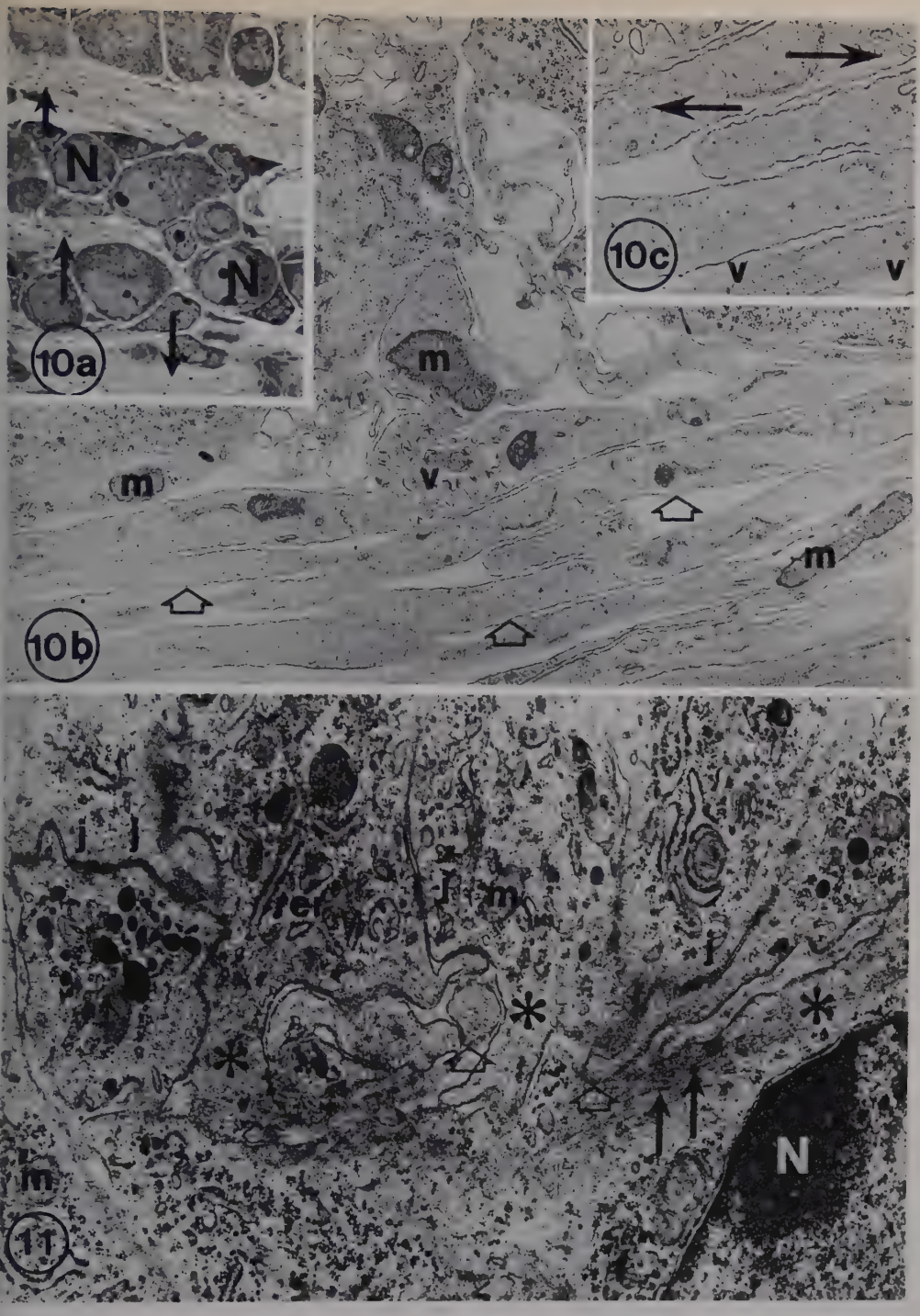


FIG. 10. (a), A light micrograph of a thick (1.0 μm) Epon section through region 2 showing large bundles f thin cellular processes (arrows). N, nuclei. × 2250. (b), An electron micrograph of the same region howing the elongated cellular processes (large arrows). These processes, after fixation in glutaraldehyde nd osmium tetroxide, are thin, averaging approximately 300 nm in diameter, and, unlike the axonal rocesses, are packed with amorphous-appearing material. m, Mitochondria; v, vesicles. × 21,000. (c), An lectron micrograph showing the structures within the cellular projections, including small vesicles (v) and vast quantity of wispy filaments (arrows). × 36,000.

FIG. 11. An electron micrograph of cells of region 2 showing a large accumulation of filamentous aterials. Numerous cells with interdigitating cellular projections (large arrows) connected by specialized ellular junctions (j), are densely packed with 70-Å filaments (asterisks) along the plasma membrane as well s within the cell body. Larger filaments, 130 Å (arrows), are also observed. m, Mitochondrion; N, nucleus; er, rough endoplasmic reticulum. × 19,000.

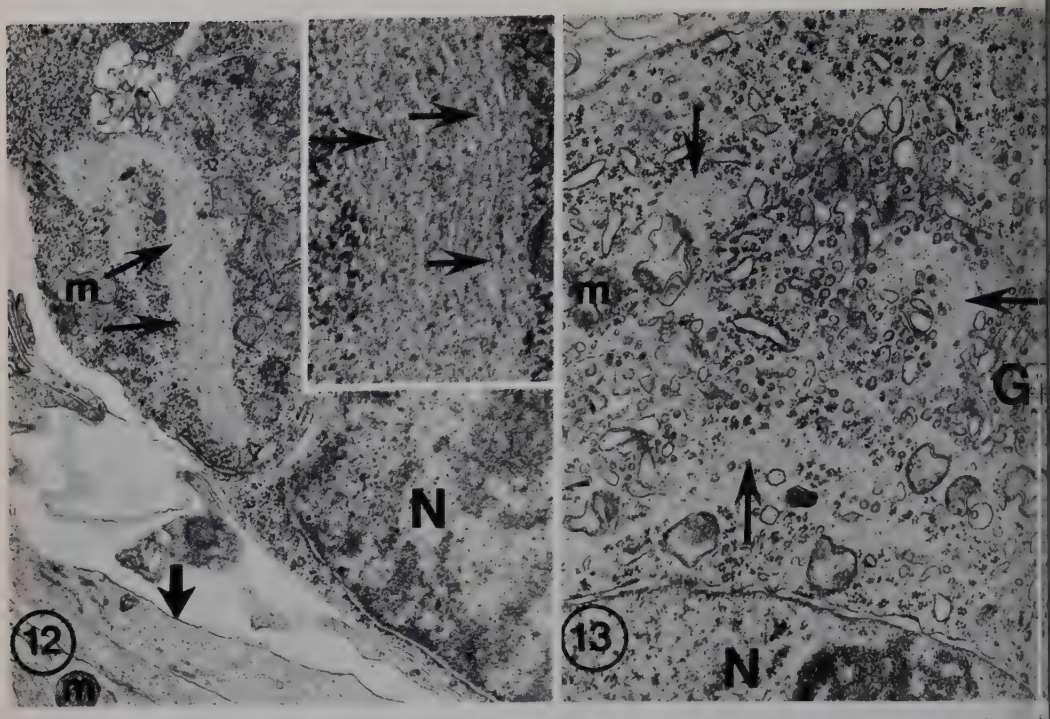


FIG. 12. An electron micrograph of region 2 after acrolein fixation. A large organized system of 100-Å filaments (thin arrows) are seen within the cell. In the lower left of the micrograph, a small bundle of cellular projections (thick arrow) is observed. m, Mitochondrion; N, nucleus. × 12,500. Inset: The tightly packed 100-Å filaments (arrows). × 44,000.

FIG. 13. A cell from region 1 after routine fixation with glutaraldehyde, containing a diffuse system of 100-Å filaments (arrows) coursing throughout the cell. G, golgi complex; m, mitochondrion; N, nucleus. × 17,000.

palatal shelf is presented in Fig. 14. Our results indicate the presence of two major contractile systems: 1) Region 1 contains well-developed as well as developing skeletal muscle fibers and is found on the oral side of the far posterior end of the palate. 2) Region 2 contains a population of filament-rich mesenchymal cells. The area of region 2 is very extensive ranging from the mid-palate on the tongue side back towards the posterior end and extends downward in this area to the limit of region 1. Finally, a third area that shows a positive myosin ATPase reaction with light-diffuse activity, termed region 3, is found along the medial edge of the palate shelf. This area encompasses the mesenchyme adjacent to the oral epithelium and extends to the mid-palate.

The orientation of the skeletal muscle fibers of region 1 was diverse, as can be

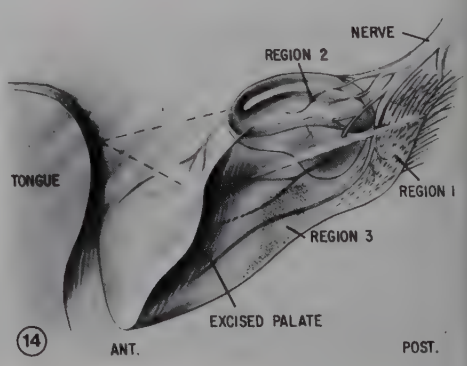


FIG. 14. Schematic drawing of the contractile and nervous systems in the vertical palate shelf. Region 1, skeletal muscle fibers; region 2, area and position of the filament-rich cells; region 3, location (stippling) of the positive myosin ATPase reaction along the oral epithelium.

seen by the cross-sectional, longitudinal and obliquely cut myofilaments present in a single section. No strict pattern of orientation could be established for the fila-

mentous system in region 2. In this region the cellular population consists of numerous elongated cells with intertwined cellular processes and shows no common alignment of filaments in adjacent cells. One possible exception may be the ordered structures observed by the myosin ATPase reaction (Fig. 3) and the bundles of tightly packed processes (Fig. 10). However, only the relative position and area of this system is indicated in Fig. 14. The extensive nerve system entering and traversing throughout the palate is also presented, although not in absolute detail. However, it should be pointed out that numerous nerve processes were observed in both regions 1 and 2. Only the presumed location of region 3, based on myosin ATPase histochemistry, is presented in the drawing. The details of this area and of the anterior palate are presently under investigation.

Pai (1965) studied the histogenesis of skeletal muscle in mouse embryos and showed that at day 13.25 of gestation large populations of myoblasts and occasional myotubes are discernible throughout the embryos, with the majority of the cells reaching the myotube stage by day 15. It is during this time of development that embryonic movements, including swallowing and neck flexion (Walker, 1969), are first observed. Biochemical evidence indicates that the day-14.5 palate, just prior to shelf rotation, synthesizes the contractile proteins actin and myosin at a rate equal to that of the tongue (Lessard *et al.*, 1974). Finally, observations presented here would seem to indicate that a skeletal muscle system potentially capable of producing a contractile force at this time of rotation is developing on the oral side of the posterior palate (region 1). In the adult human, the posterior end of the palate contains the skeletal muscles of the soft palate which function to raise the soft palate during speech and swallowing. The position of the skeletal muscle observed at day 14.5 in the mouse corresponds best to that of the palato-pharyngeal muscle in the adult human but may in fact represent the primordia of the numerous skeletal muscle fibers of the soft palate (see Figs. 22–28 in Morley, (1970)).

Similarities in the myosin ATPase reaction product of the skeletal muscle in the posterior palate (region 1) and the developing skeletal muscles of the tongue can be seen in Fig. 1. However, in region 2, the reaction product was deposited diffusely over a large area of cells (Figs. 1–3). Other investigators have reported a positive alkaline phosphatase reaction in this same general area (Sweney and Shapiro, 1971; Pourtois, 1972). Our studies (data not presented) confirm these observations. The overlap of the histochemical response may be due to a nonspecific degradation of ATP by alkaline phosphatase, indicating a false-positive reaction for myosin ATPase. An alternative explanation may be that both enzymes are present in region 2. Alkaline phosphatase, a ubiquitous enzyme, has been found associated with many processes in development, including developing contractile masses of skeletal, cardiac, and smooth muscle (Barbolini *et al.*, 1971; Kormano and Hovatt, 1972; Beckett and Bourne, 1972; Limas and Cohn, 1973). Ultrastructural examination of region 2 has revealed a large, potentially contractile population of cells containing small 70-Å filaments. It seems reasonable to assume that both enzymes, alkaline phosphatase and myosin ATPase, could be present in this region.

It is becoming increasingly clear that nonmuscle systems contribute to morphological movements during embryogenesis. Fibroblast spreading, protoplasmic streaming and axonation of neurons are just a few examples of single cell movements involving the primitive actomyosin microfilament system. Invaginations of differentiating tissues and platelet contractions might be more phylogenetically advanced, employing a nonmuscle contractile apparatus. These systems involve groups of cells. Palate shelf rotation is an example of a fairly large embryonic tissue moving from a vertical to a horizontal position. It would

seem reasonable to assume that the force needed might be greater than that produced by other systems described, yet not great enough for a skeletal muscle system. The network of filament-rich cells of region 2 possesses characteristics similar to those reported for developing smooth muscle (Yamato, 1961; Leeson and Leeson, 1965a, b; Burnstock, 1970; Uehara *et al.*, 1971; Small and Squire, 1972). These include massive amounts of small 70-Å actin filaments; thicker 130-Å myosin filaments; numerous, fine cytoplasmic processes which connect the cells with each other; and a well-developed rough endoplasmic reticulum and Golgi apparatus in the cells. Similar structures were observed in the developing smooth musculature of the embryonic gut of day-14.5 mouse embryos (data not presented). These observations suggest that the filamentous system of region 2 represents a developing smooth muscle-like mass.

Such an intermediate-type muscle system would be ideally suited for palate shelf rotation. Smooth muscle myosin shows properties very similar to nonmuscle myosin: Low ATPase activation by actin and Ca^{2+} ion dependence (Adelstein and Conti, 1972) and immunological cross-reactivity between the two myosins (Weber and Groeschel-Stewart, 1974). Development of a smooth muscle-like system in region 2 would seem to require less developmental work than a skeletal muscle system and could provide a contractile system capable of nervous stimulation and sufficient contractile force to rotate the palate shelves. However, further experiments are required before this filamentous system can be classified as a smooth muscle-like system.

A light-diffuse myosin ATPase reaction, designated region 3, was deposited in the mesenchyme just beneath the oral epithelium. Preliminary observations indicate a diffuse system of cells containing numerous 100-Å filaments and specialized cell junctions in this area (data not presented). It is of interest to note that in submucous cleft palate in humans, the midline of the palate contains well-developed skeletal muscle which contracts during phonation (Dr. Howard Aduss, personal communication). It is possible that a transient contractile system, very primitive in nature, develops in this area to aid in shelf rotation and is replaced during the normal fusion process.

The 100-Å filaments were also observed in regions 1 and 2 of the palate shelf. These intermediate-sized filaments have been reported in numerous cell types (Ishikawa *et al.*, 1968; Anderson *et al.*, 1970). The composition of these intermediate filaments is unknown but a possible relationship to microtubules is suggested by the fact that cells exposed to colchicine or colcemide exhibit a marked increase in the number of cytoplasmic intermediate filaments (Holtzer *et al.*, 1975). Further characterization of the distribution of these 100-Å filaments and the role they play in shelf rotation is the aim of future experiments.

The cross-sectional survey of the palate shelf revealed an extensive system of developing neurons, with large ganglia and associated postganglionic fibers entering into the top portion of the posterior shelf. These nerve fibers penetrate deeply into the shelf, reaching forward to near the anterior limit. The nerve fibers are unmyelinated at this time of development. In the adult human, the palate is innervated by both sensory and motor nerve fibers. The motor fibers control the soft palate musculature employed during speech and swallowing (see Morley, 1970). Similarly, nerve branches are found in the developing mouse palate in close association with both the skeletal and filamentous masses and could produce nervous stimulation of these contractile systems. From these observations a functional activity for these contractile systems can only be implied. However, experiments with whole embryo cultures show a cholinergic involvement in shelf rotation (Wee *et al.*, 1975). If these contrac-

le systems could provide the intrinsic helf force, the force might be exerted in he posterior end and produce a remodel- g of the shelf, as suggested by Coleman 965). This force could also be transferred the anterior end, which being stiffer due the developing cartilaginous rugae, ould swing into place in a barndoor fash- n.

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Cell Shape and Membrane Changes in the Eight-Cell Mouse Embryo: Prerequisites for Morphogenesis of the Blastocyst[1]

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At the eight-cell stage, the blastomeres of the preimplantation mouse embryo undergo a dramatic shape change, compaction, which is considered essential to the future segregation of presumptive cell types. This investigation demonstrates that compaction 1) occurs *in vivo*, 2) is accompanied by the formation of tight and gap junctions and 3) can be reversibly inhibited *in vitro* by calcium-depleted medium and also by cytochalasin B (CCB). Although microtubules frequently are observed in cortical regions where compaction is proceeding, colcemid and colchicine have no inhibitory effect.

Calcium-free medium and CCB dissociate compacted embryos, over 50% of which recover in normal medium in 3 and 0.25 hr, respectively. The Ca^{2+} threshold for compaction is approximately 0.1–0.2 m*M* and may be required for normal intercellular adhesions. Since compaction marks the beginning of tight junction formation and provides the necessary cell-to-cell apposition for the development of the zonula occludens at the morula stage, it is considered to be the initial step in blastocyst morphogenesis. In addition, this investigation provides the means to reprogram compaction by reversible inhibition and thereby study theories of cell determination.

INTRODUCTION

At the eight-cell stage, the blastomeres of the preimplantation mouse embryo undergo a dramatic shape change that has been considered the initial step in the segregation of inner and outer blastomeres, presumptive inner cell mass and trophoblast (Mulnard, 1967). Lewis and Wright (1935) first described this morphogenetic event in terms of the adhesion of cells to one another. The spherical shape of some blastomeres and the flattened or "compacted" appearance of others was considered to be a consequence of fundamental differences in cell surface tension and internal gelation (Lewis and Wright, 1935). More recent studies have emphasized the close membrane apposition and overlapping cellular projections of compacted blastomeres (Calarco and Brown, 1969; Calarco and Epstein, 1973).

The intracellular and intercellular events responsible for compaction remain obscure. Increased cellular apposition may be accompanied and possibly assisted by intercellular junctions. At the morula stage, compacted cells have been shown to possess gap junctions and zonular apical tight junctions (Ducibella *et al.* 1974, 1975) but their involvement in creating or maintaining cell shape is not known. The initiation of the development of zonulae occludentes may occur at the eight-cell stage (Ducibella *et al.*, 1974, 1975), possibly during compaction. In addition to junctional specializations, other forces may be operative. Cell flattening may be caused by changes in intercellular adhesive forces and/or by the involvement of intracellular microfilaments and microtubules. Therefore, this investigation has been undertaken to determine the disposition of these

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structures in the eight-cell embryo and to assess their relative roles in the process of compaction.

MATERIALS AND METHODS

Electron microscopy. At 59–66 hr post coitus, embryos were flushed from the oviducts of naturally mated Swiss mice (Charles River Breeding Labs, Wilmington, Mass.) with 3% glutaraldehyde and 0.5% paraformaldehyde in 0.1 *M* phosphate buffer (ph 7.3) at 20°C. After washing in buffer, the embryos were osmicated in 0.5 or 1.0% osmium tetroxide in buffer, rapidly dehydrated in a graded ethanol series, then infiltrated and imbedded in Epon/Araldite (Anderson and Ellis, 1965). Lanthanum impregnation was carried out by the method of Revel and Karnovsky (1967). Grey to silver sections were collected on 3% Formvar-coated 150-mesh grids and stained for approximately 30 sec in uranyl acetate (Watson, 1958) followed by lead (Sato, 1968).

Culture medium. The defined culture medium of Biggers *et al.* (1971) with 0.3% bovine serum albumin (BSA, Sigma, A-4079), was prepared according to the method of Hoppe and Pitts (1973) except that paraffin oil was not preincubated with the medium. Calcium-free medium was prepared as follows: Medium without BSA and calcium lactate was divided into two 50-ml portions. To one, designated calcium-free, sodium lactate (0.015 ml of 60% syrup) was added and to the other (normal medium) 0.026 g of calcium lactate·$5H_2O$ (Schwarz/Mann) was added. The osmolalities of the media with and without calcium were 308 and 306 mosm/l, respectively (calculated from Table 6-5 of Biggers *et al.* (1971)). BSA (0.3%) was added prior to use. Media with 1.7×10^{-5} and 1.7×10^{-4} *M* calcium were made by serial dilution of normal medium (1.7×10^{-3} *M* Ca^{2+}) with calcium-free medium. Before transfer into medium of a different calcium concentration, embryos were washed twice in that solution. *In vitro* culture was carried out in glass or 35 × 10-mm plastic dishes (Falcon #3001) under a layer of paraffin oil.

Cytochalasin B (CCB, Aldrich) and colcemid (Calbiochem) were dissolved in dimethyl sulfoxide (DMSO, Fisher) in concentrations of 5 and 1 mg/ml, respectively, and stored at 4°C. One microliter of either of these stock solutions was added to 1 ml of culture medium just prior to use. Colchicine (Fisher) was dissolved in medium to make a stock solution (100 mg/ml) which was diluted upon use. Experiments involving drug treatment were carried out in glass depression dishes filled with medium and covered with a glass coverslip. No air was allowed to remain between the medium and coverslip.

Just prior to use, all media were gassed with 5% carbon dioxide, 5% oxygen and 90% nitrogen.

In vitro culture and observations. Swiss mice were naturally mated or superovulated with 4–5 IU of pregnant mare's serum (NIAMDD[2]) and 5 IU of human chorionic gonadotrophin (Sigma). Four- to eight-cell embryos were flushed at 59–63 hr post coitum with normal culture medium.

All embryos were pooled, and as many four- to seven-cell embryos were removed as possible. Eight-cell embryos were placed in normal and experimental media and were then incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

In one experiment the zona pellucida was removed by a 5–10-min incubation at approximately 37°C in 0.5% Pronase (Sigma) dissolved in Dulbecco A phosphate-buffered saline that had been Millipore filtered before use (Mintz, 1962).

Observations were made at a magnification of 125× with a Zeiss Standard UPL inverted microscope with bright-field illumination. Eight-cell embryos whose blasto-

[2] National Institute of Arthritis, Metabolism and Digestive Diseases, Rat Pituitary Hormone Distribution Program.

here outlines were entirely separate and distinct were scored as "uncompacted." If the appositional boundaries of all eight blastomeres were not distinct, the embryo was scored as "compacted." Embryos of intermediate morphology were designated "partially compacted." The accuracy of this assay is in part a function of the magnification, clarity and resolution of the microscope used.

In order to verify the presence of eight cells in compacted embryos, cell number occasionally was monitored by counting the nuclei of embryos fixed on a slide (Tarkowski, 1966) and stained by the Giemsa procedure of Sumner (1972).

RESULTS

Embryos cultured *in vitro* undergo the same morphogenetic changes as those observed after fixation *in vivo*. Electron microscopic observations were made only on *in vivo* fixed specimens. Although development in a group of embryos is not strictly synchronous, the following observations apply to the majority of the embryos at the developmental age examined.

Normal Development

At 59 hr post coitum, embryos are at the five-to seven- or early eight-cell stages. The blastomeres of early eight-cell embryos appear approximately spheroidal and possess distinct cell outlines when observed by light microscopy (Fig. 11). Areas of cellular apposition are small and bounded by large intercellular spaces (Fig. 1).

At 62 hr post coitum, the presence of smaller spaces and increased areas of close membrane apposition indicated that cell shape changes are occurring (Fig. 12). Adjacent blastomeres begin to flatten against each other, which increases the amount of space between the embryo and the zona pellucida. Cell flattening appears to begin at initial points of cell contact and subsequently move outward. In these flattened areas, microtubules frequently appear just beneath the plasma membranes (Fig. 2; also see lower inset) and, occasionally, two or three align themselves in parallel array (Fig. 2; upper inset). At this time interdigitating microvilli and small, macular intercellular junctions develop (Figs. 5 and 6). These junctions appear below the apical areas of appositional membrane where flattening is proceeding and have a pentalaminar structure. An analysis of serial sections demonstrated that the junction in Fig. 5 was of the macular type. Focal points of apparent membrane fusion with underlying dense material develop apically, facing the zona pellucida (Fig. 7). Very few of these membrane contacts were observed, even after serial sectioning entire embryos.

Prior to the next cell division (at 64–66 hr post coitus), the eight-cell embryo is composed of a closely packed (i.e., compacted) group of blastomeres (Fig. 3). The plasma membranes of adjacent cells come in such close apposition that their boundaries become obscured when observed in the light microscope (Fig. 13), and what were initially large intercellular spaces are now restricted to small pockets. Within these small pockets endocytotic and exocytotic activity may be occurring (Figs. 3 and 4). The close membrane apposition on either side of a cleft suggests that fluid uptake or release locally overrides or changes intercellular adhesions. Microtubules continue to underlie areas of close membrane apposition (Fig. 2). In compacted embryos, two types of intercellular junctions are apparent. Focal points of apparent membrane fusion locally exclude lanthanum tracer (Fig. 8). At more basal locations, junctions present a pentalaminar appearance, lack underlying dense material, and reveal a gap of approximately 40 Å in lanthanum-impregnated specimens (Figs. 9 and 10). Mouse embryo membrane specializations of the focal type have been shown to be tight junctions, whereas those of the macu-

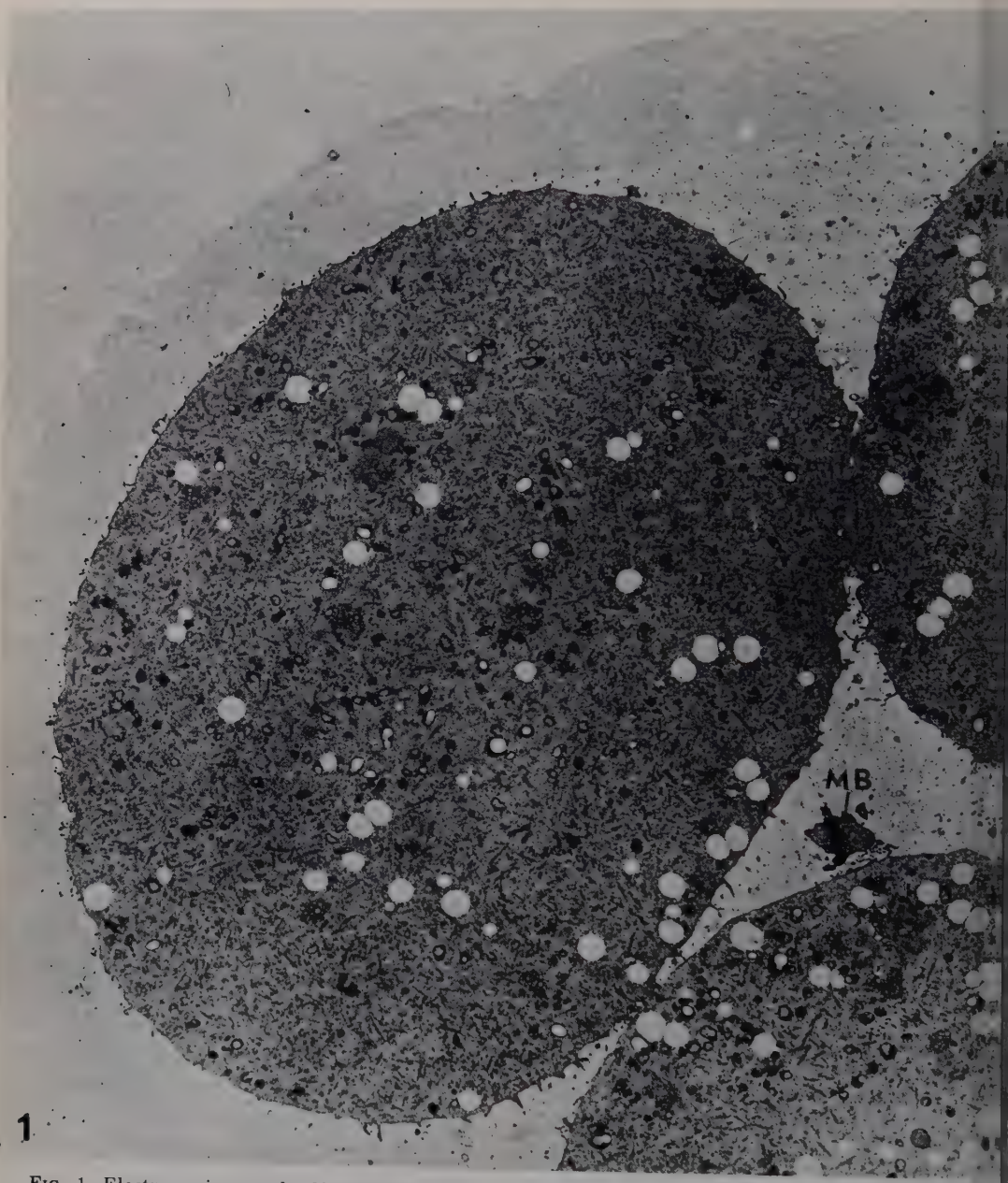


FIG. 1. Electron micrograph of blastomeres typical of early eight-cell embryos. Intercellular spaces are large and appositional membrane is minimal. Note the midbody, MB. × 3800.

lar type are gap junctions (Ducibella *et al.*, 1975). The localizations of dense material underlying tight junctions have been identified at the blastocyst stage as microfilamentlike in composition.

Development in Ca^{2+}-Depleted Medium

When embryos were cultured in calcium-free medium, compaction was completely inhibited (Fig. 14). In addition, embryos that had already compacted *in vivo* became uncompacted within 30 min following transfer to calcium-free medium (Fig. 19). In these embryos, blastomeres rounded up and remained associated at tangential points of membrane apposition. After 3 hr

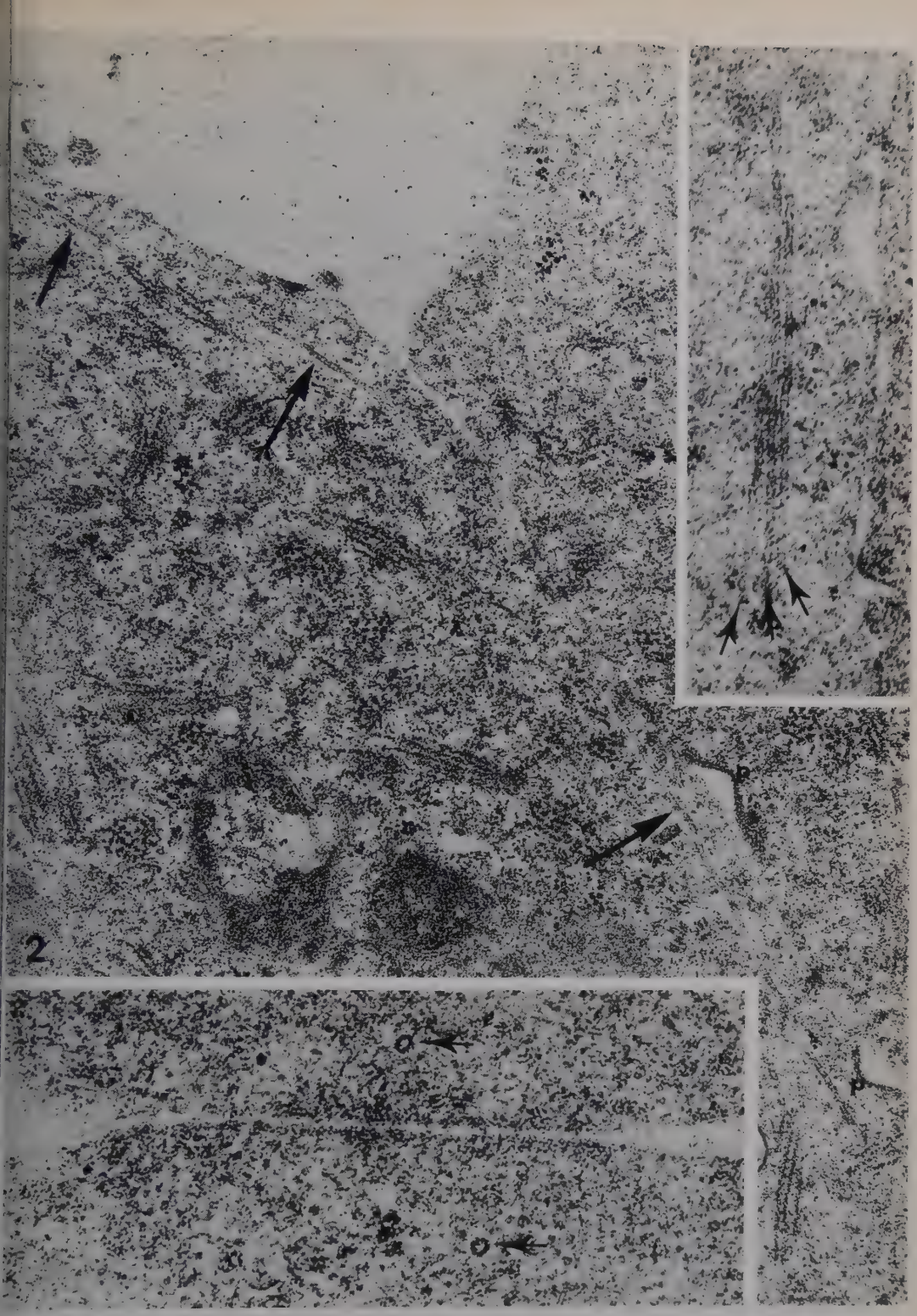


FIG. 2. Cortical microtubules (arrows) associated with the areas of close membrane apposition. In compacting embryos, microtubules are observed frequently at the cell apices where adjacent blastomeres are flattening against each other. The two small clefts in the intercellular space are caused by the formation of pinocytotic pits (p). × 42,900. In compacting embryos, microtubules are seen in cross section where membranes are closely apposed (inset, bottom, × 109,000) and in parallel arrays (inset, top. × 56,300).

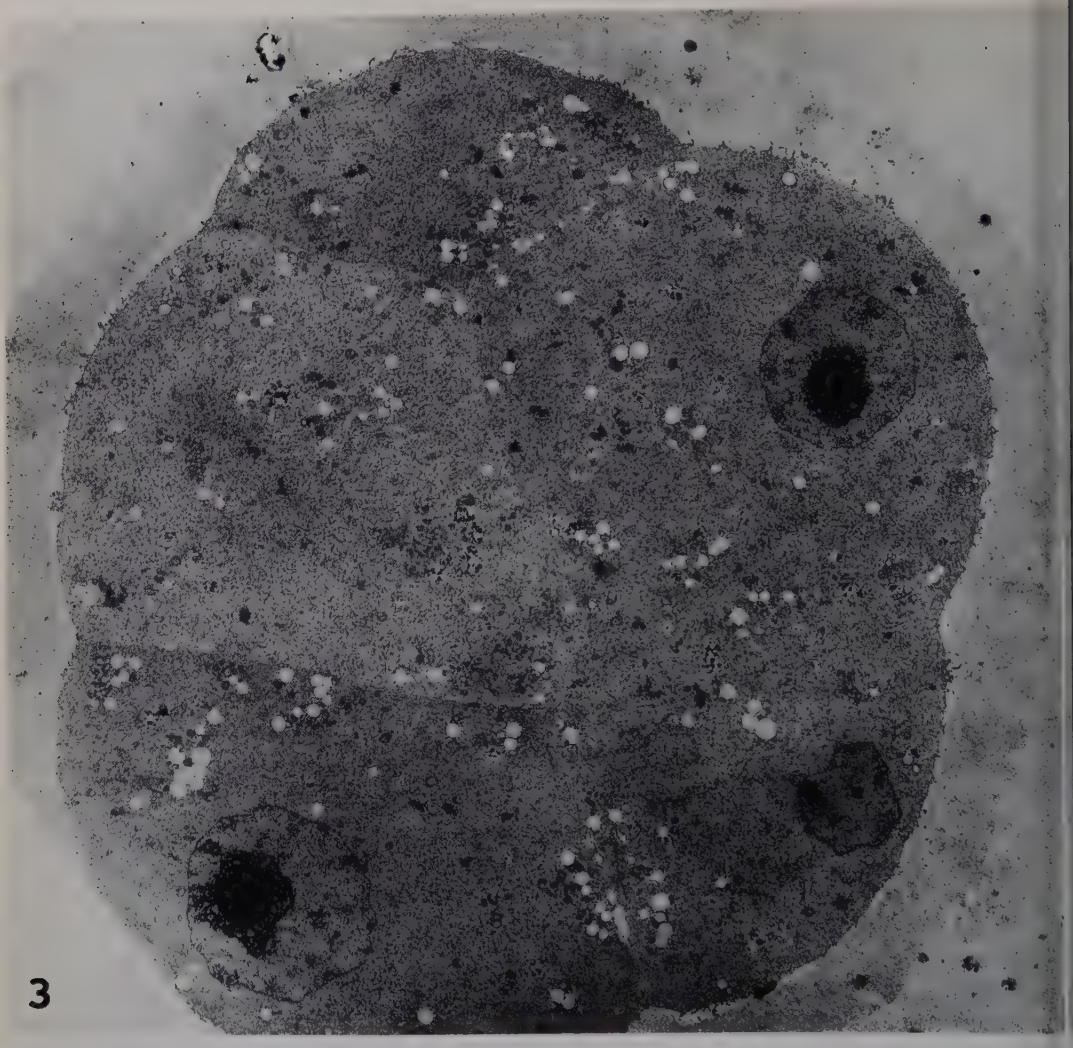


FIG. 3. Electron micrograph of a section through the center of a compacted eight-cell embryo. Note the close apposition of the cell membranes, small intercellular spaces, and absence of apical, dense microvillar localizations and junctional complexes present at the morula stage. × 2400.

in culture, a large majority of embryos had completed compaction in normal medium, whereas all of those in calcium-free medium were uncompacted (Table 1; also see Figs 14 and 15). When the latter were transferred into normal medium, they proceeded to compact (Fig. 16). Further, those embryos that had compacted *in vitro* became uncompacted when placed in calcium-free medium (Fig. 19). When pulsed with calcium-free medium, embryos demonstrated the ability to undergo two cycles of compaction and retained the ability to compact at least 3 hr after control embryos had completed compaction. Embryos that were pulsed in calcium-free medium for at least 3 hr were able to undergo compaction at a faster rate than unpulsed controls. For example, 43% of the control embryos underwent compaction during the first 1.5 hr of the experiment, whereas the two groups of pulsed embryos rescued in normal medium were 68 and 70% compacted after 1.5 hr (calculated from the data of Fig. 19). Since about half of the embryos in the control group were partially compacted

TABLE 1

THE EFFECT OF VARIOUS TREATMENTS ON THE COMPACTION OF EIGHT-CELL EMBRYOS *in Vitro*

Treatment	Time (hr)	Percentage compacted at the end of treatment	
		Initially not compacted[a]	Initially compacted
Normal medium	3	68% (38)[b]	100% (39)
Calcium-free medium	3	0% (52)	0% (39)
DMSO (1 μg/ml)	2.5	69% (26)	100% (30)
Cytochalasin B[c] (5 μg/ml)	2.5	0% (11)	0% (30)
Colcemid[c] (7.5 μg/ml)	3	69% (13)	100% (8)
Colchicine (1 μg/ml)	3	72% (18)	
2°C	1		100% (17)

[a] Uncompacted or partially compacted.
[b] Number of embryos scored.
[c] Stock in DMSO; see Materials and Methods.

at the beginning of the experiment and all were uncompacted in the pulsed group, the difference in compaction rates is much greater.

The calcium concentration threshold required for compaction was also investigated. Figure 20 demonstrates the effect of different calcium ion concentrations on groups of embryos that were 45–55% compacted at the beginning of treatment. A 100-fold decrease in calcium concentration was 97% effective in preventing embryos from attaining or maintaining the compacted state. At 1.7×10^{-4} *M*, there was a small inhibitory effect, slowing the rate of compaction. Thus, the apparent extracellular calcium threshold is approximately 10^{-4} *M*.

The Effects of Cytochalasin B, Colcemid, and Colchicine

Since microtubules and microfilaments have been implicated in cell shape changes, the effects of CCB, colcemid, and colchicine on compaction were investigated.

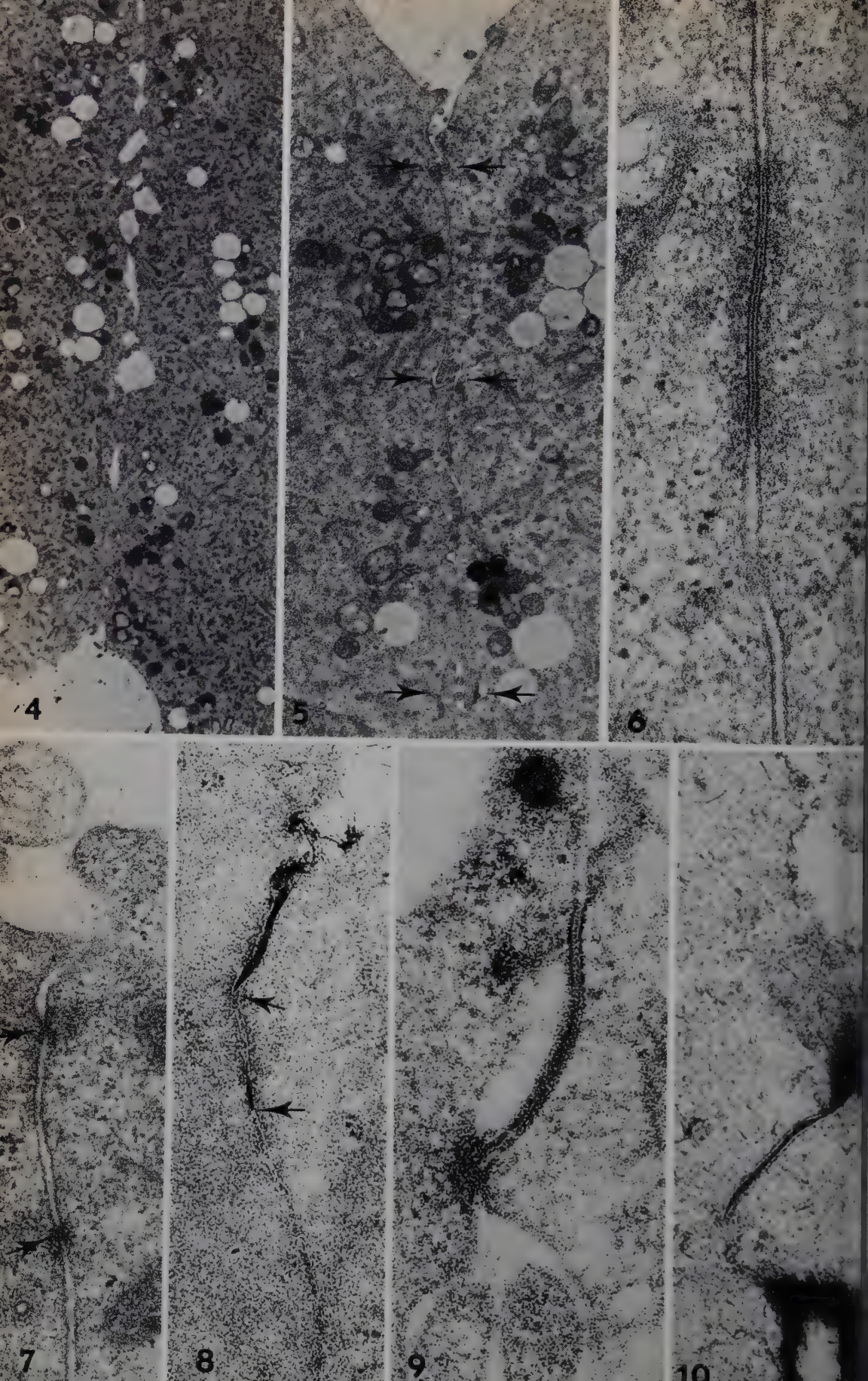
CCB at a concentration of 5 μg/ml prevented compaction and caused all of the blastomeres of compacted embryos to round up within 1.5 hr (Fig. 17; Fig. 21). After 5 hr in CCB over 80% of the embryos transferred to DMSO control medium completed compaction in 15 min (Fig. 21); at 0.5 μg/ml, compaction was not inhibited (Fig. 18). DMSO (1 μg/ml) had no detrimental effect at the light microscopic level.

In the presence of colchicine (1 μg/ml) and colcemid (7.5 μg/ml dissolved in DMSO), embryos underwent compaction at the normal rate and those initially compacted were not affected (Table 1). Low temperature, which also depolymerizes cytoplasmic microtubules, had no effect on compacted embryos after a 1-hr incubation (Table 1).

DISCUSSION

This study demonstrates that compaction at the eight-cell stage of the mouse embryo occurs *in vivo*, is accompanied by the formation of tight and gap junctions and can be reversibly inhibited *in vitro* by lowering the Ca^{2+} concentration of the medium or by CCB. These data are summarized in Fig. 22. Although calcium-free medium and CCB reverse compaction at the light microscopic level, the fates of the junctions, microtubules, and filaments are under investigation.

The formation of apical tight junctions during compaction (62 hr post coitum) marks the beginning of the development of the zonula occludens at the morula stage. The impermeability of the zonula occludens to lanthanum tracer at 70 hr post coitum (Ducibella *et al.*, 1974, 1975) indicates that zonular tight junctions develop over a period of approximately 8 hr in Swiss albino mice. In the uncompacted state, a zonula occludens can not develop because only macular areas of cell–cell apposition are present. Only focal or nonzonular tight junctions could form, and these have not been observed. Close membrane apposition at apical sites (facing the zona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ellucida) appears to be a requirement for ight junction ontogeny. Therefore, we iew the phenomenon of compaction as a rerequisite not only for the segregation of nner and outer cells at the morula stage Mulnard, 1967; Tarkowski and Wrobewska, 1967) but also for the establishnent of the zonula occludens. This permeaility barrier is necessary for the accumulaion of the blastocoel fluid and may play a ole in cell determination by creating a nicroenvironment within the embryo (Duibella *et al.*, 1974, 1975).

The infrequency with which tight and gap junctions were observed and the abence of adhering junctions (e.g., desmoomes) suggest that other forces are responible for the close membrane apposition of ompacted embryos. Calcium-dependent ntercellular adhesive forces may be operaive. The calcium requirement for cell adhesion in early mouse embryos has been demonstrated previously by Wales (1970) and by Whitten (1971). When embryos were cultured from the two-cell stage in 0.1 m*M* and 0.03 *M* calcium-containing medium, the percentages of embryos forming blastocysts (compared to controls) were 33%[3] and 0%, respectively (Wales, 1970). Our data on the effects of calcium depletion on compaction compare favorably; the percentages of partially and completely compacted embryos in 0.17 and 0.017 m*M* Ca^{2+} are 85 and 0%, respectively. Thus, the similar Ca^{2+} thresholds for blastocoel formation and compaction, approximately 0.1–0.2 m*M*, support the contention that compaction is a prerequisite for the formation of the blastocoel. There appears to be no analogous requirement for Mg^{2+} since 1) there is only a small decrease in the percentage of embryos forming blastocoels in Mg^{2+}-free medium (Wales, 1970) and 2) increased [Mg^{2+}] will not substitute for decreased [Ca^{2+}] (Brinster, 1964).

The importance of Ca^{2+} in intercellular adhesion has been reviewed by Steinberg (1958, 1962) and by Manery (1966). Calcium ions are required for the adhesion of many embryonic and epithelial cell types. Curtis (1962) has observed that *Xenopus laevis* embryonic cells dissociated in calcium-depleted medium reaggregate when their surfaces become resaturated with Ca^{2+}. The saturation level, 0.2 m*M*, is similar to that required for compaction and blastocoel formation. Steinberg (1958) also has demonstrated the reversibility of dissociation in calcium-free media and the greater effectiveness of Ca^{2+} in promoting reaggregation than other divalent cations. Calcium may act as an intercellular link, for example, by coupling anionic groups of apposing cell membranes to themselves or to a divalent intercellular adhesive glycoprotein (Steinberg, 1962; Culp, 1974; Kuhns *et al.*, 1974). In addition, studies on artificial membrane bilayers provide evidence that Ca^{2+} binds to phospholipid moieties and changes in Ca^{2+} concentration can cause isothermal lipid phase transitions (Papahadjopoulos *et al.*, 1973; Trauble and

[3] Calculated from the data of Wales (1970), Table 1.

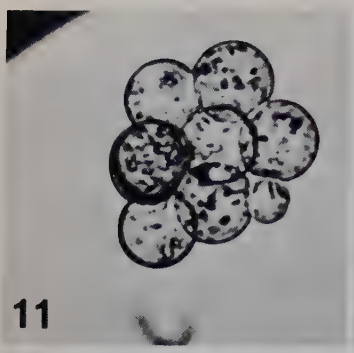
FIG. 4. Dilated intercellular clefts possibly created by exocytotic and endocytotic activity. × 8500.

FIG. 5. Interdigitating microvilli (arrows) of two blastomeres of a compacting embryo. × 17,600.

FIG. 6. Newly formed junction between two blastomeres of a compacting embryo. × 111,000.

FIGS. 7 and 8. Apical tight junctions (arrows) between blastomeres which have flattened against each other. Figure 7 demonstrates two areas of apparent membrane fusion with underlying dense material and Fig. 8 is a similar section of a lanthanum impregnated embryo. Although the tracer is occluded locally (arrows), it is able to gain access to the interior of the embryo. Figure 7, × 70,700. Figure 8, × 72,700.

FIGS. 9 and 10. Gap junctions between blastomeres of compacted embryos. These have not been observed apically and are found in deeper regions of cellular apposition. Note the absence of dense material subjacent to the junctional membrane and approximate 40-Å gap upon lanthanum impregnation. Fig. 9 (× 120,000). Fig. 10 (× 120,000).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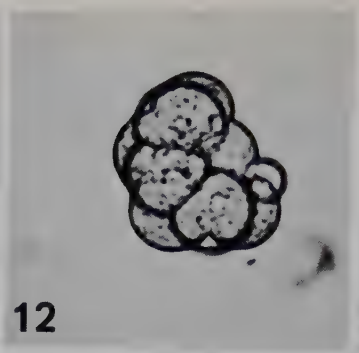
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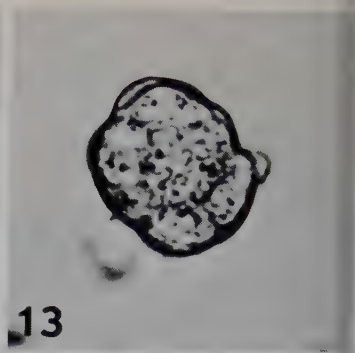
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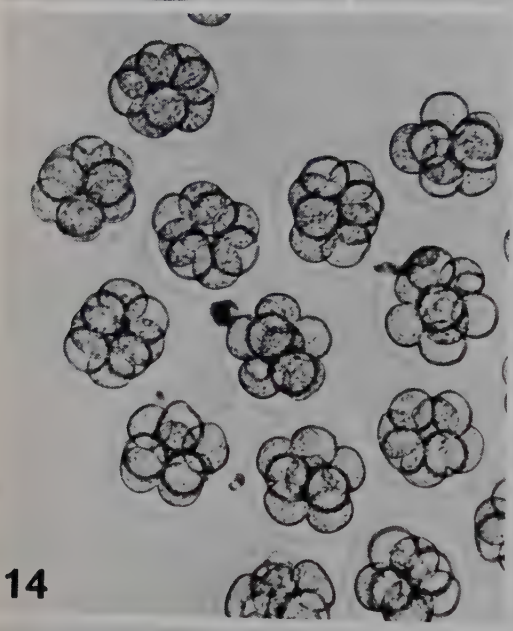
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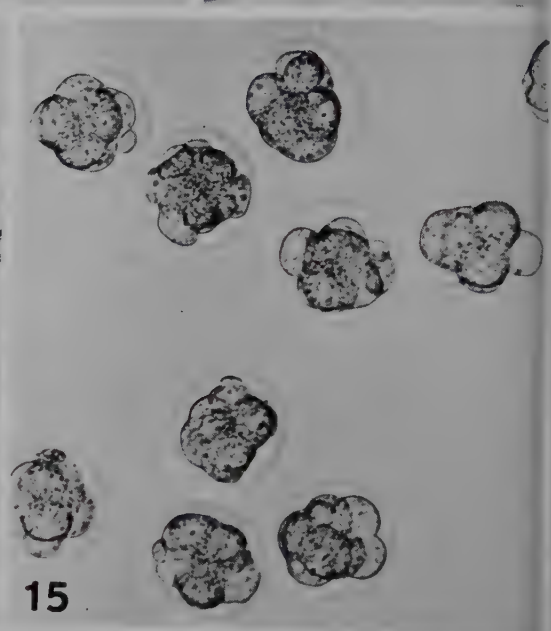
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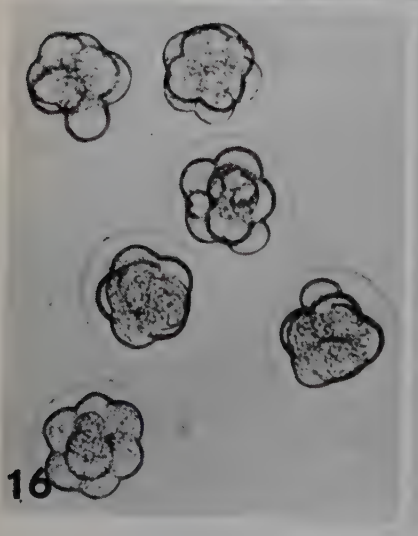
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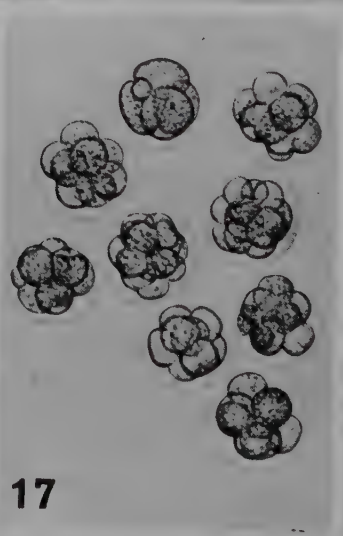
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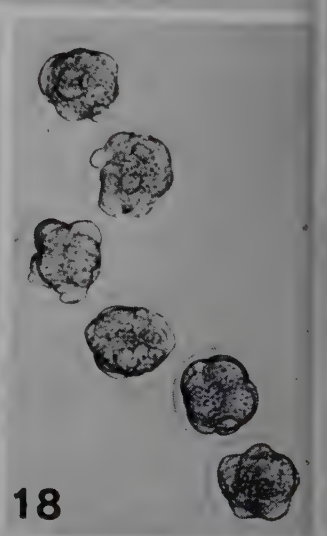
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Gibl, 1974). Extracellular Ca^{2+} also has a significant effect on cell permeability (Mayery, 1966) and neural cell surface sialidase activity (Schengrund and Nelson, 1975).

Compaction may be due to a change in cell–cell adhesion during the eight-cell stage or a change in the deformability of the membrane or both. In a compacted embryo, the blastomeres appear to maximize cell–cell contact. During the early eight-cell stage, an increase in intercellular adhesive forces could cause cell flattening, minimize the total adhesive free energy (Steinberg, 1963), and lead to the most stable equilibrium configuration, i.e, the compacted state.The same final state could be reached if cell membrane deformability (e.g., pseudopodiallike activity) increased. The rounding up of cells during mitosis is characteristic of embryonic cleavage stages (Rappaport, 1973) and presumably accounts for the spheroidal blastomere shape at the early eight-cell stage. Compaction may occur passively by a relaxation of those constraints present dur-

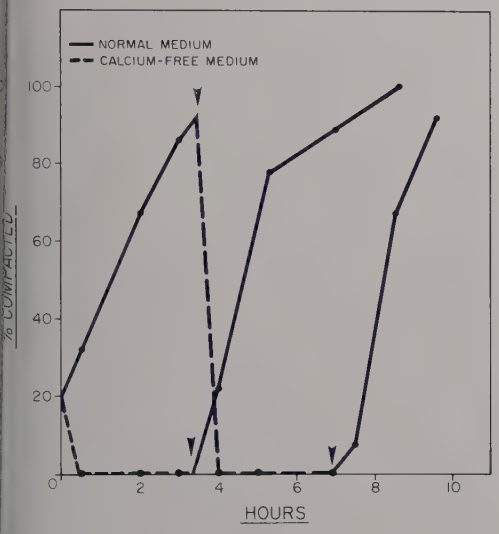

FIG. 19. Graph of a pulse experiment with calcium-free and normal media. Seventy-four embryos were pooled in normal medium and separated into two groups, each with 20% compacted. During the first 3.5 hr, one group was incubated in calcium-free medium (dashed line) and the other group in normal medium (solid line). The arrows indicate when the calcium-free-treated embryos were transferred into normal medium and those in normal medium transferred into calcium-free medium. The initial rate of compaction in the calcium-free-pulsed embryos is approximately twice that of the unpulsed controls (see Results).

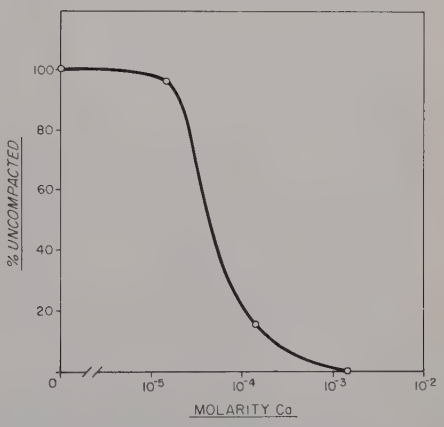

FIG. 20. The effect of different calcium concentrations on compaction. Embryos were pooled in normal medium and subsequently transferred to either 0, 1.7×10^{-5}, 1.7×10^{-4}, or 1.7×10^{-3} *M* (normal) medium. Each point represents at least 30 embryos of which 45–55% were initially compacted and is an average from at least two experiments.

FIGS. 11, 12, and 13. Living eight-cell embryos in different stages of compaction. Figure 11 demonstrates the uncompacted condition. The blastomeres of the embryo in Fig. 12 are flattening against each other, and in the completely compacted embryo (Fig. 13) cell boundaries are obscure or indistinguishable. For enzymatic removal of their zonae, these embryos were processed simultaneously in the same dish of Pronase solution. The small cell at the lower right of each figure is the polar body.

FIGS. 14, 15, and 16. Eight-cell embryos from a single experiment in which one group was pulsed with calcium-free medium; 20% were compacted at the beginning of treatment. Fig. 14, embryos in calcium-free medium after 2 hr. Fig. 15, control embryos in normal medium after 2 hr. Fig. 16, calcium-free-pulsed embryos after rescue in normal medium for 0.75 hr.

FIG. 17. Uncompacted embryos in CCB (5 μg/ml) after 2.5 hr.

FIG. 18. Compacted embryos in CCB (0.5 μg/ml) after 2.5 hr.

ing mitosis and/or actively by cells sending out projections over neighboring cells. Scanning electron microscopy has dramatically demonstrated such processes (Calarco and Epstein, 1974) and, further, cell spreading becomes a frequent occurrence at the morula stage (Ducibella *et al.*, 1975). Cell spreading and contact inhibition of locomotion in tissue culture cells is accompanied by microfilament bundle assembly subjacent to close cell–cell contacts (Goldman *et al.*, 1974). A somewhat analogous process occurs during compaction: As cell spreading is completed, apical focal tight junctions with underlying filamentous material form and, subsequently, cell locomotion appears to cease.

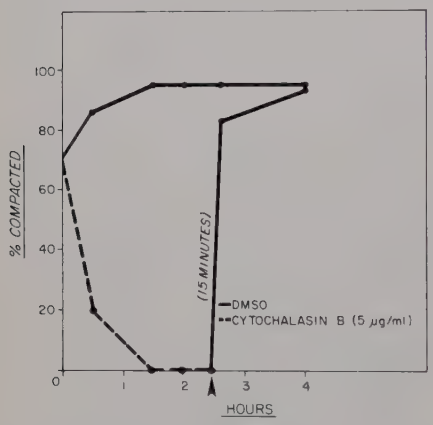

FIG. 21. The effect of CCB (5 μg/ml) on compaction. The protocol was similar to that described in Fig. 19. Each point represents at least 20 embryos, and in two experiments more than 80% of the embryos in CCB (dashed line) recovered within 15 min after transfer to normal medium.

Since cell locomotion appears to require a functional microfilament or actomyosin system (Pollard and Weihing, 1974), the inhibition of compaction by CCB is suggestive of active cell spreading movements. Although the mode of action of CCB has not been elucidated, the drug has been found to bind to plasma membrane fractions (Mayhew *et al.*, 1974) and may act by interfering with the association of actin with the plasma membrane (Spooner, 1973). Cell spreading may be minimally a two-step process: 1) Short cell processes are extended and subsequently 2) undergo a calcium-dependent adhesive attachment on the surface of an adjacent blastomere. Compaction may proceed by the continued repetition of these steps, but the finding that CCB dissociates a completely compacted embryo is unexpected unless the drug affects sites on the outside as well as the inside of the membrane. Schaeffer *et al.* (1973) have presented evidence that CCB induces a change in the cell surface charge density of some, but not all, of the cell types of the amphibian embryo. At 5 μg/ml, amphibian cells round up, dissociate completely in 1.5 hr, and the process is reversible; this is the same cellular behavior demonstrated by the blastomeres of CCB-treated, compacted mouse embryos. Bluemink (1971) has reported that close intercellular contacts in the amphibian embryo are disrupted after CCB treatment. At later stages of development in the

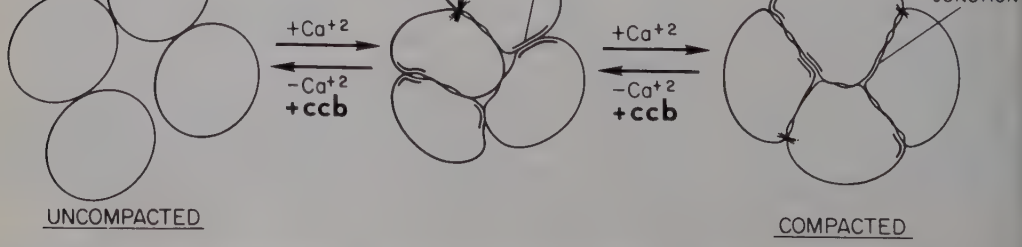

FIG. 22. Schematic representation of compaction and the effects of calcium-free medium and CCB (see Discussion).

lick, there is evidence that CCB inhibits ll motility to a greater extent than cell hesion (Armstrong and Parenti, 1972; einberg and Wiseman, 1972; Overton d Culver, 1973). Unlike CCB, colcemid DMSO) and colchicine had no effect, hich suggests that microtubules are not quired for compaction.

Since compaction appears to be the ini- al step in blastocyst morphogenesis, it ay be characteristic of all higher mam- alian embryos. Photomicrographs of liv- g monkey and rabbit embryos demon- rate that compaction occurs by the 16- ll and morula stages, respectively (Lewis d Hartman, 1933; Adams, 1970). Earlier servations provided evidence that close ll packing begins at the eight-cell stage the rabbit (Assheton, 1894; Lewis and regory, 1929). If in the rabbit, compac- on and early junction formation are also lcium dependent, the use of calcium-free edium should be avoided in tracer stud- s of intercellular junctions (Hastings and nders, 1975). In the mouse, compaction, ke blastocoel formation, appears to be a evelopmentally programmed event sched- led in time but not with respect to cell umber (Tarkowski, 1959; Ducibella and nderson, unpublished observations). his study provides the means to repro- ram compaction by reversible inhibition nd to study the determination of presump- ive inner cell mass cells. It should be possi- le to discern whether cell position inside he morula is the only prerequisite for de- ermination or, in addition, if the proper ntercellular junctions and microenviron- nent of the compacted embryo are re- uired.

We thank Steven Dizio and Richard Tasca for haring their observations on cytochalasin-treated mbryos, Paul Burgoyne for his invaluable advice on *n vitro* culture techniques, and David Albertini, ohn Biggers, and Wesley Whitten for helpful dis- ussions.

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Maturation of Rabbit Reticulocytes: Degradation of Specific Reticulocyte Proteins

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As analyzed by polyacrylamide-gel electrophoresis, rabbit reticulocyte cytoplasm contains, in addition to globin, seven predominant polypeptides. The amounts of these range from 0.1 to 1.2% of globin. Rabbit erythrocytes contain only three of these nonglobin polypeptides. The loss of the four other polypeptides is correlated with maturation of the reticulocytes to erythrocytes. We also fractionated reticulocytes according to age by equilibrium centrifugation through a gradient of metrizamide and showed that younger reticulocytes contain much more of these four polypeptides than do more mature reticulocytes.

The four polypeptides that are lost during differentiation are also very sensitive to *in vitro* destruction by chymotrypsin or trypsin under conditions where globin and the three reticulocyte nonglobin peptides that remain during reticulocyte maturation are completely resistant. Our results are consistent with the notion that the degradative rate of a reticulocyte cytoplasmic protein is determined by its susceptibility to general intracellular proteases.

INTRODUCTION

Both the rate of degradation and the rate of synthesis of a specific protein are important in determining its concentration in a cell. The classic work of Schimke and co-workers (1965), for instance, showed that the increased level of tryptophan pyrrolase found in rat liver after administration of tryptophan is due to enhanced stability of the enzyme, not to an increased rate of synthesis. There exist several excellent reviews on intracellular protein degradation (Goldberg and Dice, 1974; Schimke, 1970, 1973, 1974; Rechcigl, 1971; Pine, 1972).

The degradation rates of different proteins within animal cells can vary over a wide range. Essentially all proteins of rat liver take part in a continual replacement process, and liver proteins have an average half-life of 3.5 days (Schimke, 1970). The half-life of individual enzymes, however, varies from 11 min for ornithine decarboxylase (Russell and Snyder, 1971) to 19 days for isozyme-5 of lactate dehydrogenase (Fritz *et al.*, 1973). Even different subunits within a multimeric protein may have distinct half-lives (Fritz *et al.*, 1973). The same enzyme may have different half-lives in different tissues (Segal *et al.*, 1969; Fritz *et al.*, 1969).

In general, polypeptide chains of higher molecular size are degraded more rapidly than smaller ones (Dehlinger and Schimke, 1971, 1972; Dice *et al.*, 1973; Gurd and Evans, 1973; Goldberg and Dice, 1974). The rate of degradation of a protein *in vivo* correlates well with its rate of degradation by specific proteases, such as chymotrypsin or Pronase, *in vitro* (Goldberg, 1972; Dice *et al.*, 1973; Goldberg *et al.*, 1974). The simplest explanation of the diversity of protein half-lives would be that the degradative rates are determined by the susceptibility of a protein to general intracellular proteases (Goldberg, 1972; Goldberg and Dice, 1974). Supporting this hypothesis, it was found that mutant proteins, or proteins containing amino acid analogs, have a less than normal half-life and are also more susceptible to degradation *in vitro* by purified proteases (Goldberg, 1972; Goldberg and Dice, 1974).

Study of protein turnover in most cells is complicated by continual protein synthesis and by reutilization of isotopes from de-

graded enzymes for synthesis of new proteins. Reticulocytes are an ideal system for studies of protein and messenger RNA degradation. They have no nucleus and make no RNA. They are active in protein synthesis, but over 90% of the protein produced is a single species, hemoglobin (Lodish and Desalu, 1973).

In this paper we show that reticulocytes contain seven major polypeptides in addition to globin. Only three of these proteins are found in mature erythrocytes, and decreasing amounts of the other four proteins are found in reticulocytes of greater maturity. We show, further, that the four polypeptides that are preferentially degraded in the reticulocyte are also more sensitive *in vitro* to digestion by proteases.

MATERIALS AND METHODS

Phenylhydrazine-anemic rabbits. New Zealand white rabbits weighing 4–6 lb were given subcutaneous injections for 5 days of 2 ml of 1.2% acetylphenylhydrazine. On the 7th day the animal was bled from the ear. As determined by staining with methylene blue, over 90% of the blood cells were reticulocytes.

Phlebotomized-anemic rabbits. On day 1, 2.0 ml of acetylphenylhydrazine was injected subcutaneously, and 40 ml of blood was removed from an ear. On each day following, 40–50 ml of blood was taken from one ear. On days 3 and 6 the rabbit was also given an intramuscular injection of 1.0 ml of iron-dextran (50 mg of Fe/ml, Imferon, Lakeside Laboratories, Milwaukee, Wis.). By day 10 generally over 80% of the blood cells were reticulocytes.

Preparation of cell supernatant fractions. Blood was collected in 50 ml of solution A (0.14 *M* NaCl, 0.0015 *M* magnesium acetate, 0.005 *M* KCl) containing 0.001% heparin. The cells were collected by centrifugation at 4000 rpm for 5 min and washed by centrifugation three times in solution A. The buffy coat containing white cells was removed after the first two centrifugations. The packed cells were lysed in 5.6 volumes of 5P8 [0.005 *M* sodium phosphate, pH 8 (Fairbanks *et al.*, 1971)] and centrifuged at 15,000 rpm for 20 min. The supernatant fluids were stored frozen until used. Hemoglobin was quantitated by absorbance at 540 nm of a dilution of the supernatant fluid in Drabkin's solution (Wintrobe, 1974).

Metrizamide gradients. An aqueous metrizamide solution (Gallard–Schlesinger Corp., Carle Place, N. Y.) of 34.77% (w/v) is isotonic. A 14.6-ml linear gradient was made in a tube for 1 Beckman SW 27.1 rotor. The mixing chamber (27% metrizamide) contained 5.67 ml of the isotonic metrizamide solution and 1.63 ml of solution B (0.118 *M* NaCl, 0.005 *M* KCl, 0.0167 *M* Na_2HPO_4, 0.00063 *M* $CaCl_2$, 0.00179 *M* $MgSO_4$, 4% bovine serum albumin, pH 7.5); the reservoir (12% metrizamide) contained 2.52 ml of isotonic metrizamide solution and 4.78 ml of solution B. Washed cells from a phlebotomized-anemic rabbit were resuspended in five volumes of solution B, and 3.0 ml of this were layered atop this gradient. In some experiments the cells were labeled *in vitro* with [^{35}S]methionine for 1 hr (Lodish and Desalu, 1973). Centrifugation was at 15,000 rpm at 4°C for 1 hr in a Beckman ultracentrifuge. The gradients were collected from the bottom of each tube by a peristaltic pump.

Gel electrophoresis. Aliquots of the supernatant fluids were added to the urea–sodium dodecyl sulfate–phosphate gel buffer utilized previously (Lodish and Desalu, 1973) and placed in a boiling-water bath for 2 min. They were analyzed by electrophoresis through cylindrical 7.5% polyacrylamide gels containing 6 *M* urea and 0.1% sodium dodecyl sulfate, as detailed previously (Lodish and Desalu, 1973). Staining of the gels with Coomassie blue and destaining followed the procedure of Fairbanks *et al.* (1971), except that no Coomassie blue was used in the destaining solution. The gels were scanned at 560 nm in a Gilford spectrophotometer.

RESULTS

ytoplasmic Proteins in Reticulocytes and Erythrocytes

Polyacrylamide-gel electrophresis in the resence of 6 *M* urea and 0.1% sodium odecyl sulfate resolves rabbit reticulocyte toplasm into eight polypeptide species peaks I–VII and globin, Figs. 1A and E). ssentially the same profile was obtained om reticulocyte-rich blood taken from rabbits made anemic by phenylhydrazine injection (Fig. 1A) or by daily bleeding (Fig. 1E). Approximate molecular weights of these proteins are given in the legend to Fig. 1. None of these polypeptides is a ribosomal protein; when reticulocyte cytoplasm was centrifuged for 2 hr at 100,000*g* to pellet ribosomes and the supernatant fluid analyzed, patterns of polypeptides identical to those shown in Figs. 1A and E

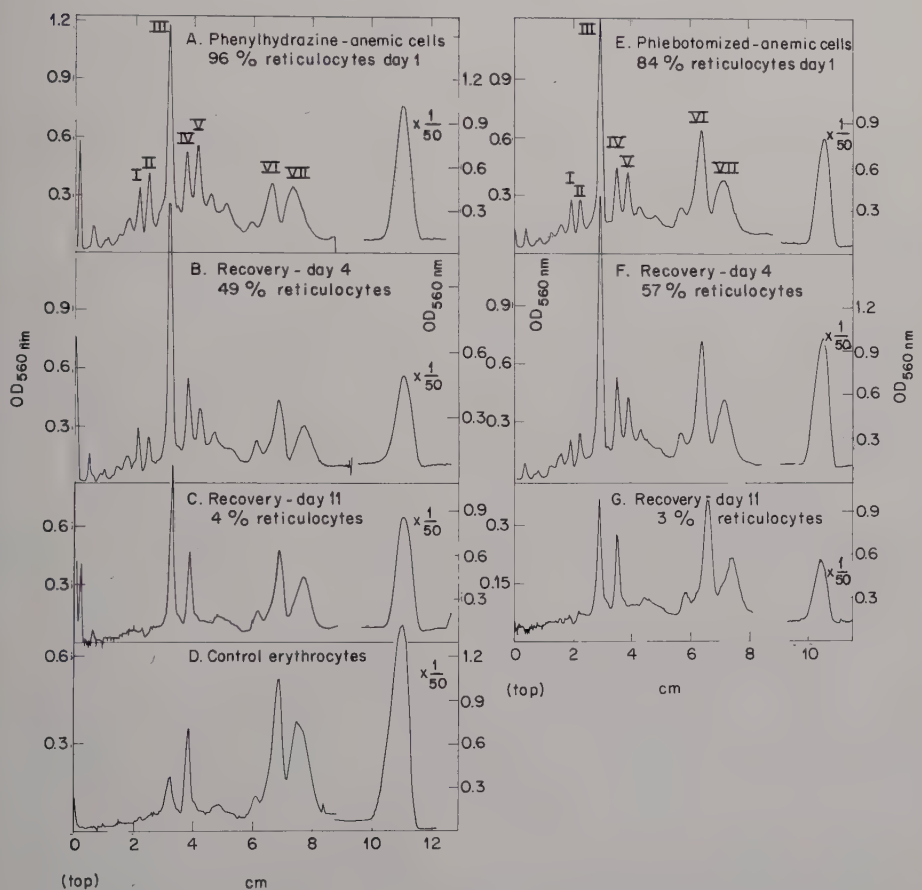

FIG. 1. Polyacrylamide gel electrophoresis of cytoplasmic extracts of reticulocytes and erythrocytes. Two abbits were utilized, one made anemic by injection with phenylhydrazine (A–C) and one made anemic by leeding (E–G); see Materials and Methods. The control erythrocytes used in (D) were from the same rabbit sed for phenylhydrazine-induced anemia and were taken before the phenylhydrazine injections began. For oth anemic rabbits, day 1 is the day of maxium reticulocytosis; see Materials and Methods. The animals ere allowed to recover, with no further injections or massive bleedings. At 4 days (B and F) and 11 days (C nd G), about 5 ml of blood were withdrawn and analyzed. Two samples of each cytoplasmic extract were nalyzed by gel electrophoresis; the main part of each graph is a spectrophotometer tracing of a Coomassie lue-stained gel used to analyze 10 μl of supernatant fluid, while the tracing of the globin region was of a gel hat contained 0.2 μl (A–C, E–G) or 0.33 μl (D) of cell supernatant. Approximate molecular weights of the even labeled polypeptides were derived by coelectrophoresis with a series of standard proteins (Lodish and Desalu, 1973): I, 79,000; II, 74,000; III, 64,000; IV, 57,000; V, 52,000; VI, 33,000; VII, 29,000.

were obtained. As determined from the intensity of staining by Coomassie blue, the amounts of the seven minor polypeptides ranged from about 1% that of globin (band III) to 0.1% of globin (bands I and II) (Fig. 2, day 1). Note that in Fig. 1 each cytoplasmic extract was analyzed on two gels; the one used for quantitation of globin contained only 2–3% the amount of extract as the one used to quantitate the other even polypeptides.

Cytoplasm from erythrocytes, by contrast, does not contain polypeptides I, II, or V (Fig. 1D). It contains essentially the same amount of species IV, VI, and VII, relative to globin, as that of reticulocytes (Fig. 2) and contains markedly reduced amounts of polypeptide III.

As measured by absorbance at 540 nm in Drabkin's solution (Wintrobe, 1974), the amount of hemoglobin per reticulocyte is within 10% of that of an erythrocyte: $2.84 \pm 0.10 \times 10^{-11}$ g/cell for the preparation used in Fig. 1A, $2.83 \pm 0.09 \times 10^{-11}$ for that in Fig. 1E, and $2.93 \pm 0.13 \times 10^{-11}$ for the control erythrocytes used in Fig. 1D. Hence each erythrocyte contains less than 15% of the amount of polypeptides I, II, and V found in a precursor reticulocyte (cf. Figs. 1A and D).

Figures 1 and 2 also show the results of experiments in which anemic rabbits were allowed to recover. By day 4, only about half of the peripheral blood cells stained as reticulocytes with new methylene blue; the supernatant fluids from these cells contain only about half the initial amount of polypeptides I, II, and V (Figs. 1B and F). By day 11, few reticulocytes remain in the blood; such cells contain no detectable amounts of polypeptides I, II, or V (Figs. 1C and G). These results support the notion that polypeptides I, II, and V and, to a lesser extent, III are lost during maturation of reticulocytes to erythrocytes.

Fractionation of Reticulocytes by Metrizamide Gradient Centrifugation

Further correlation between reticulocyte maturation and the loss of these polypeptides requires fractionation of a population of reticulocytes according to age. To this end we centrifuged a sample of blood cells from a phlebotomized rabbit (85% reticulocytes) through a linear gradient of metrizamide. The cells had been previously labeled *in vitro* with [^{35}S]methionine, since younger reticulocytes are more active in protein synthesis than older ones. Figure 3 shows that younger cells (high ratio of counts per minute per cell) band at a lighter density than do more mature reticulocytes, as is the case as well in gradients of bovine serum albumin (Glowacki and Millette, 1965). The ratio (counts per minute per cell) increases monotonically across the gradient.

Figures 4 and 5 show that the younger reticulocytes (fractions E and F) contain much more of polypeptides I, II, and V, relative to globin, than do the more mature cells in fractions A or B. The younger cells also contain sixfold more of polypeptide III than do the older cells. Since the amount of globin per cell is the same in all six metrizamide fractions (Fig. 3), we conclude that maturation of reticulocytes is indeed correlated with loss of polypeptides I, II, V and, to a lesser extent, species III.

Digestion of Reticulocyte Proteins by Purified Proteases

As discussed in the Introduction, the rate of degradation of a protein *in vivo* often correlates with its sensitivity to degradation by purified proteases *in vitro*. Accordingly, it was of interest to determine whether reticulocyte proteins that are lost during maturation (I, II, III, V) are more sensitive to proteases than those that are not degraded during maturation (IV, VI, VII, globin). The experiment depicted in Fig. 6 shows that indeed this is the case. Following treatment of a reticulocyte extract with 200 μg/ml of α-chymotrypsin, polypeptides I, II, and V are lost, and III is present in reduced amounts. Polypeptides IV, VI, VII, and globin are unaffected. Higher levels of chymotrypsin result in further loss of species III and also of VII

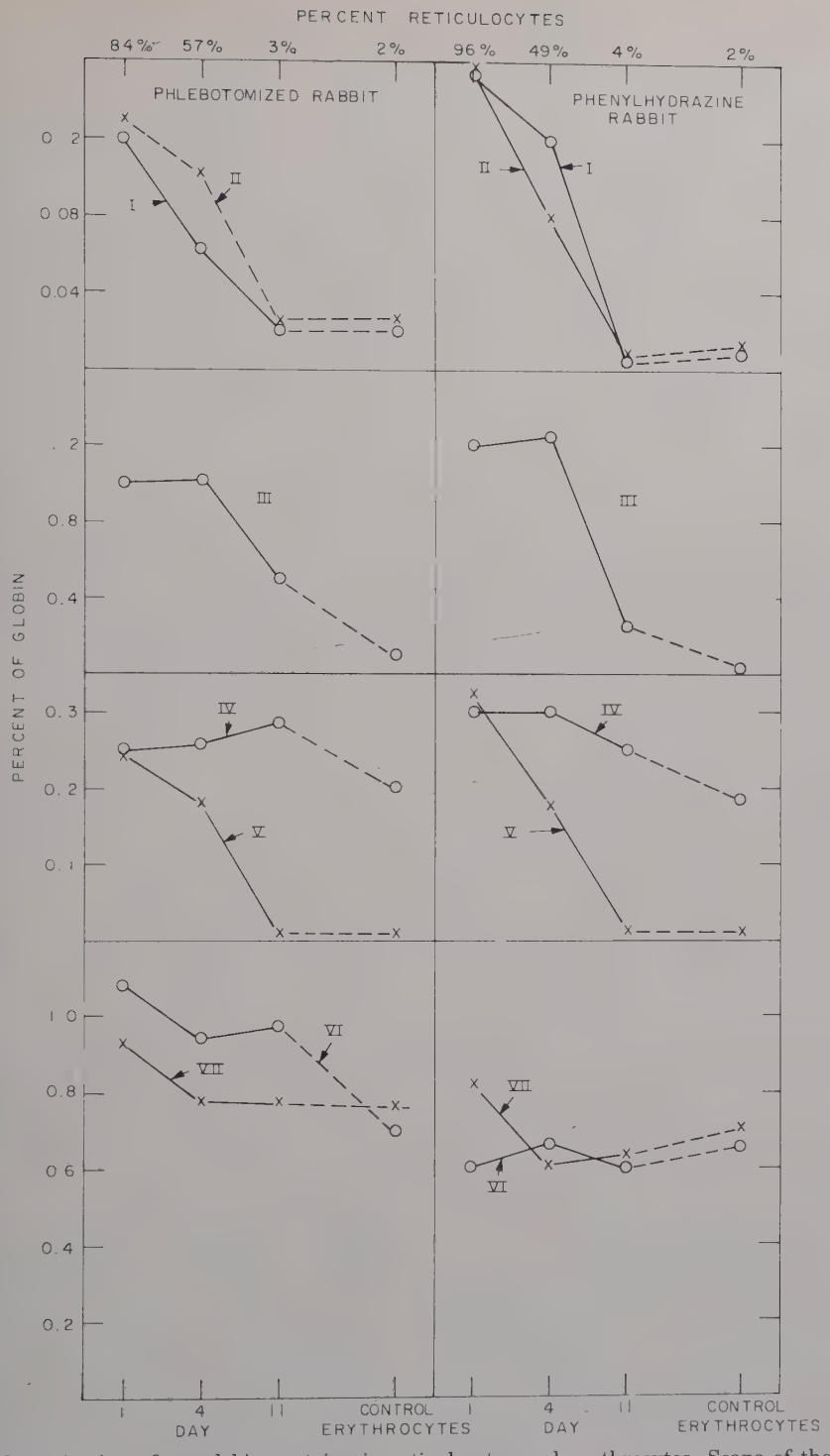

FIG. 2. Quantitation of nonglobin proteins in reticulocytes and erythrocytes. Scans of the polyacrylamide gels used are those of Fig. 1, except that the erythrocytes used as control for the phlebotomized rabbit were taken from the same rabbit 3 weeks after the last bleeding, at which time the peripheral blood contained the normal 2% reticulocytes. The areas under the eight principal polypeptides were determined with a planimeter and normalized to the corresponding area under the globin peak. An arbitrary baseline was established by connecting with straight lines all of the troughs in the optical density profiles; hence, the absolute but not the relative amount of each of the proteins is subject to some error.

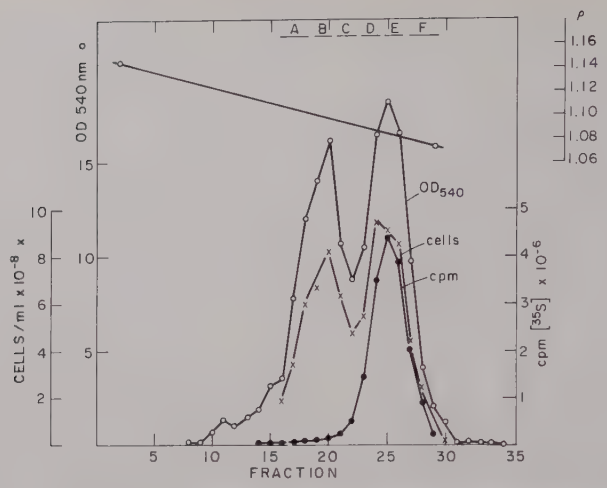

FIG. 3. Separation of reticulocytes by centrifugation through a metrizamide gradient. A sample of cells from a phlebotomized rabbit, containing 85% reticulocytes, was labeled *in vitro* with [^{35}S]methionine and then centrifuged to equilibrium in a gradient of metrizamide. Both the $OD_{540\ nm}$ and radioactivity are for the entire initial 1.0-ml fraction. Density was determined by weighing aliquots in a calibrated micropipet. The percentage of reticulocytes in each pooled fraction was determined after washing the cells free of the metrizamide and albumin solutions: Fraction A, 8%; B, 18%; C, 40%; D, 77%; E, 98%; F, 98%.

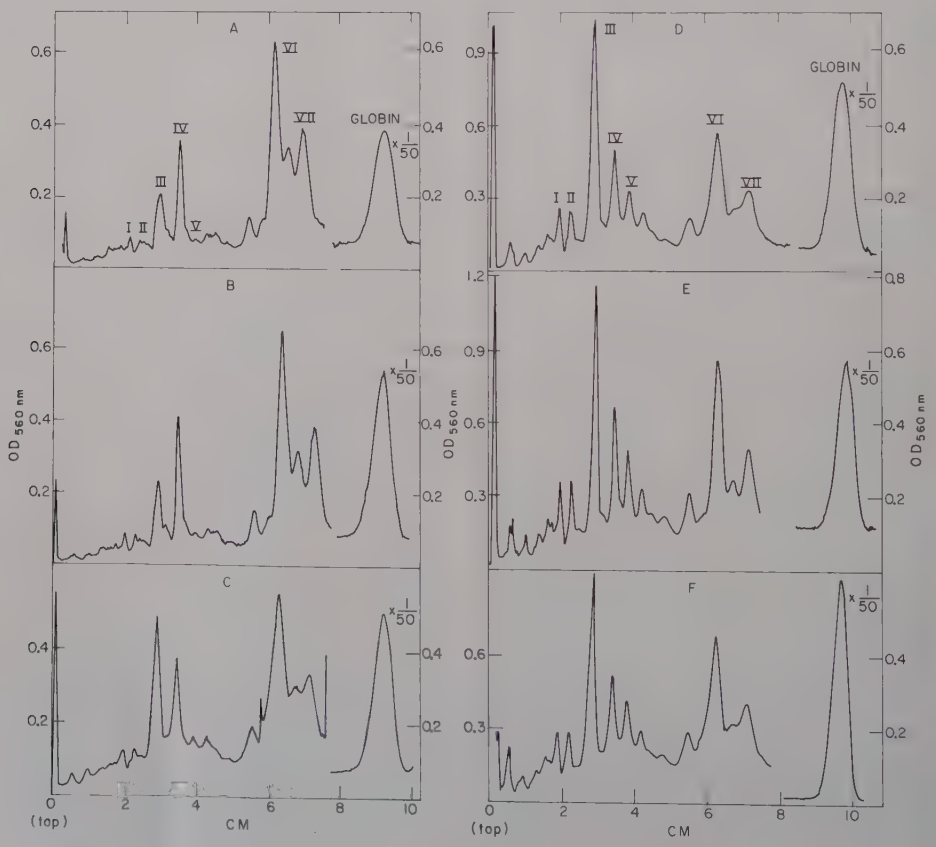

FIG. 4. Polyacrylamide-gel electrophoresis of cytoplasmic extracts of reticulocytes fractionated on a metrizamide gradient. The letters A–F refer to the six samples taken from the gradient depicted in Fig. 3.

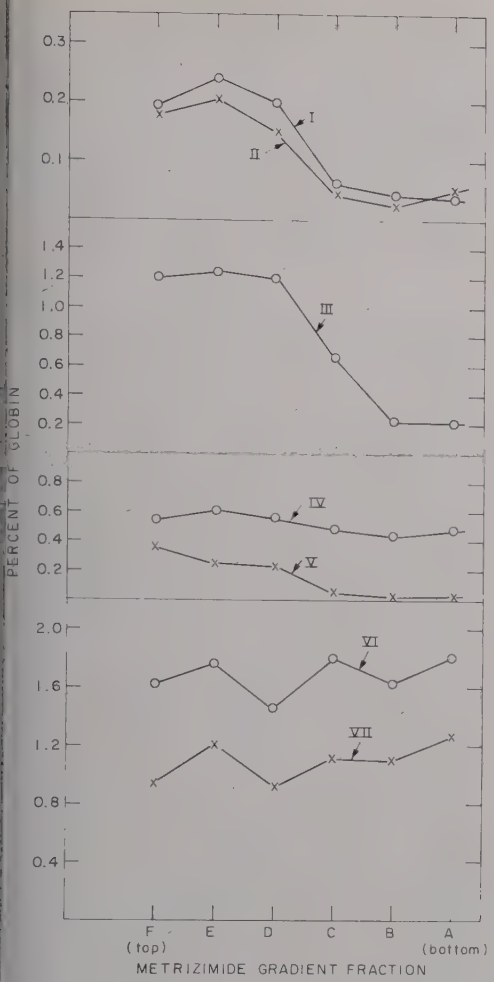

FIG. 5. Quantitation of nonglobin proteins in the cytoplasm of reticulocytes fractionated on a metrizamide gradient. The tracings of the polyacrylamide gels depicted in Fig. 4 were analyzed as described in the legend to Fig. 2.

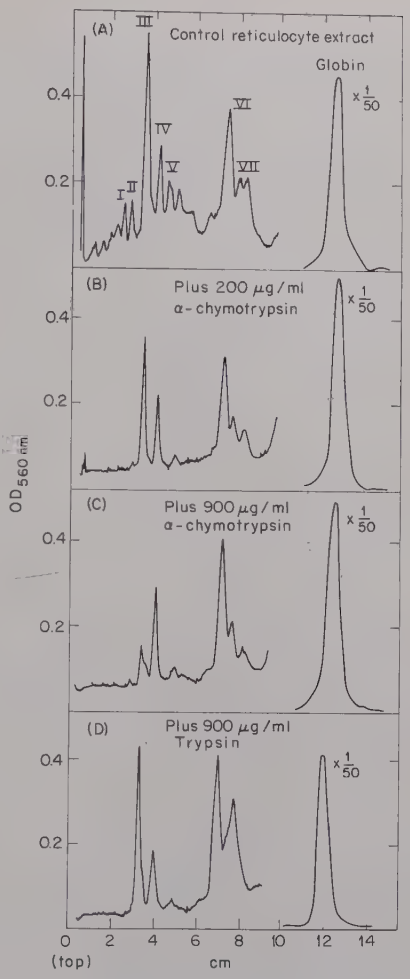

FIG. 6. Digestion of reticulocyte proteins with chymotrypsin or trypsin. To 100 μl of a cytoplasmic extract of reticulocytes (rabbit made anemic by phenylhydrazine) were added: (A), 9 μl of water; (B), 2 μl of α-chymotrypsin (10 mg/ml); (C), 9 μl of α-chymotrypsin (10 mg/ml); (D), 9 μl of trypsin (10 mg/ml). The samples were incubated at 37°C for 10 min. Aliquots of 10 μl, used to quantitate the non-globin proteins, and 0.2 μl, used to quantitate the globin region of the gel, were added to 100 μl of the urea–SDS solution used for gel electrophoresis and immediately boiled for 2 min. Polyacrylamide gels were run and analyzed as in Figs. 1 and 4.

ut leave IV, VI, and globin unaffected. rypsin also digests polypeptides I, II, and ; in this case proteins III, IV, VI, VII, nd globin are not degraded at all. We onclude that there is an excellent correlaion between degradation of reticulocyte roteins *in vivo* and *in vitro*; polypeptides , II, and V, which are completely lost uring differentiation, are destroyed by rypsin or chymotrypsin under conditions here polypeptides IV, VI, VII, and globin, which are not lost during differentiaion, are unaffected. We cannot draw any irm conclusions about peptide III, since its sensitivity to trypsin and chymotrypsin is different.

DISCUSSION

Reticulocytes are the penultimate cell in erythropoiesis. They contain no nucleus and make no RNA. They do contain many ribosomes. Although about 90% of the protein produced is hemoglobin, reticulocytes

do make several discrete nonglobin proteins (Lodish and Desalu, 1973) including two membrane proteins (Lodish, 1973; Lodish and Small, 1975). Normally, reticulocytes are confined to the marrow and mature into erythrocytes within 1 day. During severe anemia, reticulocytes are prematurely released into the peripheral blood and may comprise as much as 90% of the circulating red blood cells.

Several techniques can fractionate a population of reticulocytes according to age, including non-water-miscible fluids of known specific gravity (Danon *et al.*, 1965) and equilibrium sedimentation in gradients of bovine serum albumin (Leif and Vinograd, 1964; Glowacki and Millette, 1965), dextran (Schulman, 1067), and stractan (Corash *et al.*, 1974). In this paper we show that gradients of metrizamide, an iodine-containing derivative of glucose, also separate reticulocytes according to age. Metrizamide offers several advantages over these other techniques: 1) A high density is achieved with low osmolarity, and hence these gradients do not alter the osmotic pressure in the cells; 2) the gradients have a high capacity for cells; and 3) they cost less, are more reproducible, and are easier to prepare than gradients of serum albumin.

Maturation of reticulocytes is accompanied by loss of ribosomes. More mature reticulocytes contain fewer ribosomes than young cells, and a smaller fraction of the ribosomes are found on polysomes and are active in protein synthesis (Danon *et al.*, 1965; Glowacki and Millette, 1965; Marks *et al.*, 1963). Maturation is also accompanied by loss of one or more protein factors required for initiation of protein synthesis (Herzberg *et al.*, 1969). Reticulocytes also contain one or two membrane proteins not found in erythrocyte membranes and which are presumably lost at some stage in maturation (Koch *et al.*, 1973; Lodish and Small, 1975).

We show here that reticulocytes contain at least three predominant nonglobin protein species that are not ribosomal proteins and that are lost during maturatio to erythrocytes. Older reticulocytes co tain much less of these proteins than younger reticulocytes (Figs. 3–5).

The enzyme(s) responsible for degrad tion of these proteins is unknown. O results are consistent with the notion th the degradative rate of a reticulocyte cyt plasmic protein is determined by the su ceptibility of the protein to general intr cellular proteases (Goldberg, 1972; Gol berg and Dice, 1974). The four reticulocy polypeptides that are found at the sam amount in erythrocytes (bands IV, VI, VI and globin, Fig. 1) are largely resistant *in vitro* degradation by chymotrypsin trypsin (Fig. 6). By contrast, the three pol peptides that are lost during maturatio (bands I, II, and V) are destroyed rapid by these enzymes. Band III, which is al lost during maturation, but at a slow rate than bands I, II, or V (Fig. 5) is d stroyed by chymotrypsin *in vitro*, but at slower rate than are I, II, and V (Fig. 6 The significance of this last result clouded by the finding that band III resistant to degradation *in vitro* by tryps (Fig. 6). It will be of some interest to is late proteases from reticulocytes and te their action on the seven predominant no globin polypeptides.

Our results are not in strict accord wi the notion that subunit protein size is major determining factor in protein stab ity (Dehlinger and Schimke, 1971, 197 Dice *et al.*, 1973; Goldberg *et al.*, 197 While, in general, larger reticulocyte pol peptides turn over more rapidly than smaller ones, one polypeptide (IV) that stable during maturation is larger tha one (V) that is completely lost. Differenc in the protein composition of reticulocy and erythrocyte cytoplasm is, we conclud due to two processes: Synthesis of speci nonglobin proteins by reticulocytes (Lo ish and Desalu, 1973) and degradation certain nonglobin proteins during matur tion.

We are grateful to Dr. Fred Goldberg of Harva Medical School for his important suggestion that

determine the effects of proteases on reticulocyte cytoplasmic proteins. This work was supported by grants No. AI-08814 and AM-15929 from the U.S. National Institutes of Health. H.F.L. is a recipient of Research Career Development Award No. GM-50175, also from the N.I.H. H.C. is supported by Public Health Service Training Grant No. AM-05581-07 at Children's Hospital, Boston, Mass.

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Programmed Synthesis of Collagen during Postembryonic Development of the Nematode *Panagrellus silusiae*

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The relative rate of collagen synthesis in the free-living nematode *Panagrellus silusiae* during postembryonic development was found to be discontinuous by measuring either the incorporation of tritium into material extracted as collagen or the amount of collagen-bound tritiated proline and hydroxyproline after 2-hr incubations of whole worms with [^{3}H]proline. A peak of collagen production preceded each of the three molts that were examined. Moreover, protocollagen prolyl hydroxylase activity during each intermolt period paralleled the pattern of collagen synthesis. On the other hand, a triphasic pattern was not observed when noncollagenous proteins were labeled with either [^{3}H]tryptophan or [^{3}H]leucine. In addition, the level of soluble radioactive proline that accumulates in whole organisms after 2-hr incubation periods did not fluctuate appreciably during postembryonic development. The mean ratio of hydroxyproline to proline in a number of collagen samples extracted at various times during the maturation phase was 0.113 ± 0.040. Pulse and chase experiments with [^{3}H]proline indicated that most of the collagen synthesized during a peak period is lost after the second ecdysis following the labeling interval. In contrast, a considerable proportion of the collagen synthesized during nonpeak periods is retained throughout the postembryonic period. It is postulated that the modulated pattern of collagen biosynthesis in *Panagrellus* reflects, for the most part, a quantitative regulation of the production of cuticular collagen during postembryonic development.

INTRODUCTION

The entire exoskeleton (cuticle) of nematodes is replaced four times during postembryonic development. In general terms each of these molts includes two phases: 1) The complete assembly of a new cuticle from diverse precursors and 2) the subsequent sloughing off of a more or less intact cuticular complex. Neither the molecular compostion nor the organization of the nematode cuticle is well known. Fine structure studies of various nematode species indicate a plethora of cuticular types that range from elaborate multilayered structures to simple organized aggregates (Bird, 1971). Despite this diversity, a generalized nematode cuticle is thought to consist of three basic longitudinal zones: The cortical, median and basal layers. In different nematodes and to varying extents, each layer has specialized elaborations.

The major constituent of the nematode cuticle is protein (Bird and Bird, 1969) which the prevalent representative is collagenlike molecule. In one well-studi nematode, the proportion of collagen in t intact cuticle is problematic. For examp estimates of 6% (Chvapil and Ehrlic 1970), 50% (Bailey, 1968) and 95% (Fu moto and Kanaya, 1973) have been ported for the relative collagen content the whole *Ascaris* cuticle. The princip collagen found in the *Ascaris* cuticle h been isolated and characterized (McBri and Harrington, 1967; Harrington a Rao, 1970; Harrington and Karr, 19 Hauschka and Harrington, 1970a,b,c). T relatively high proline content (about 30 and low hydroxyproline concentrati (about 1.6%) and the presence of disulfi bonds make this collagen singular, a though, in other properties, it resembl vertebrate collagen. Discrete collage have also been identified in muscle, g

nd reproductive tissues of *Ascaris* (Fujiioto, 1968; Rudall, 1968; Cain, as cited by ain and Fairbairn (1971)). To date, comarative studies on nematode collagens ave not been conducted. Within *Ascaris*, ody wall (muscle) collagen differs from uticular collagen in a number of aspects. riefly, muscle collagen has a higher hyroxyproline content, lower proline concenation, fewer half-cystine residues and igher carbohydrate content than cuticur collagen (Fujimoto, 1968). In addition, ne collagen of the muscle layer is not eadily extracted with dilute salt soluons. It has been suggested that the *Asiris* muscle collagen is similar to the enal basement membrane collagen of igher vertebrates (Fujimoto, 1968).

The imino acid hydroxyproline is found n few kinds of polypeptides (Matthews, 967; Sadava *et al.*, 1973) of which, in anials, collagen comprises the major repreentative. Therefore, operationally, the resence of radioactive hydroxyproline in rotein extracts of whole worms after incuation with labeled proline can be used as n indicator of the biosynthesis of collagen. his approach would not measure exclusively the synthesis of cuticular collagen. lowever, it can be argued that information about the pattern of cuticular collagen roduction can be obtained without the olation of the cuticle per se if cuticular ollagen comprises the preponderant type f collagen in the organism. In nematodes his condition seems to hold. For example, n *Ascaris*, an organism from which pure uticles can be readily isolated, about 40% f the total body weight is due to cuticle, ypodermis and muscle, and 82% of the ollagen found in these components is deived from the cuticle (Chvapil and Ehrch, 1970). Similarly, we have found that bout 66% of the total polypeptide-bound yrrolidine of *Ascaris* is in the cuticle (unublished). Consequently, in those nemaodes from which the cuticle is not easily olated the incorporation of radioactive proline into protein that is extracted as collagen should, to a limited extent, provide an index of the biosynthesis of the major type of collagen, i.e., cuticular collagen.

As yet it has not been determined whether the assembly of the new cuticle in nematodes is dependent upon the induction of specific biosynthesis of various cuticular components prior to each molt or if the constituents of the replacement cuticule are produced continuously throughout each intermolt period. There are suggestive data supporting both notions. At the time of molting there is a marked increase in the biosynthetic activity in the tissue underlying the cuticle (Kan and Davey, 1968; Samoiloff and Pasternak, 1969; Lee, 1970; Bonner and Weinstein, 1972). As well, Cain and Fairbairn (1971) have found a large rise in protocollagen prolyl hydroxylase activity at the first molt in *Ascaris*. On the other hand, since there is considerable growth between molts in nematode species, it is assumed that cuticular components are produced continuously. Recently, evidence of the incorporation of proline into the cuticle of *Ascaris* after the final molt demonstrated that the synthesis of some cuticular components is not correlated with molting (Fujimoto, 1967; Fujimoto and Prockop, 1969; Chvapil, *et al.*, 1970). These studies complement the earlier work of Watson (1965) who observed that the cuticle of *Ascaris* increases threefold in thickness after the final molt.

Regardless of the definitive aspects of the process, the occurrence of successive molts in nematodes has potential merit as a representative system for the examination of the temporal regulation of the synthesis of various macromolecular components and their subsequent assembly into an organized array at specific times during the life cycle. In this regard, a study of the molting process in the free-living nematode *Panagrellus silusiae* was facilitated because it is possible to synchronize its

postembryonic development (Westgarth-Taylor and Pasternak, 1973). For these reasons, an examination of the pattern of collagen synthesis throughout the postembryonic development of *P. silusiae* was undertaken. Specifically, this paper is concerned with the following questions: 1) What is the relative rate of collagen synthesis during postembryonic development? 2) Is collagen biosynthesis a continuous or discontinuous process? 3) To what extent is collagenous material conserved from one molt to the next?

MATERIALS AND METHODS

The free-living nematode *Panagrellus silusiae* was grown xenically in 4.5% Czapek Dox agar at room temperature. Worms at the L2 stage (about 300 μm in length) were isolated from mixed populations, washed and cultured for synchronous postembryonic growth (Westgarth-Taylor and Pasternak, 1973). Growth was monitored by measuring the lengths of worms that had been heat fixed in lactophenol–cotton blue at various times during the duration of the experiment (Samoiloff and Pasternak, 1969).

At regular intervals samples containing 200 worms were taken from synchronously growing cultures and incubated with L-[5-^{3}H]proline (Amersham/Searle; 5 Ci/mmol; 5–20 Ci/ml) at 5 μCi/ml for 2 hr. After each pulse, the organisms were thoroughly washed free of unincorporated label and stored in 5% trichloroacetic acid at 5°C. In similarly designed experiments, L-[^{3}H]tryptophan (G) (Amersham/Searle; 2 Ci/mmol; 10 Ci/ml) or L-[4,5-^{3}H]leucine (Amersham/Searle; 250 mCi/mmol; 10 Ci/ml) was used in place of [^{3}H]proline. In a series of preliminary experiments, it was found that removal of the food organisms prior to the labeling period did not alter the subsequent pattern of incorporation.

About 0.5 ml of unlabeled worms was added to each sample of 200 labeled worms to provide carrier material. The augmented samples were washed three times in cold 5% trichloroacetic acid. The firs pellet was resuspended in 1 ml of distill water and sonicated at 4°C with six 20-s bursts of a Branson Ultrasonicator set 80 W. The homogenates were made 5 with respect to trichloroacetic acid a heated at 90°C for 60 min to extract coll gen (Fitch, *et al.*, 1955). After centrifug tion at 2000*g* both the pellet (noncollag nous protein) and the supernatant flu (collagenous protein) were dialyz against distilled water at 5°C overnigh Both extracts were adjusted to 6 *N* H and hydrolyzed in sealed evacuated tub for 12–16 hr at 120°C. The hydrolyzat were evaporated to dryness and the re dues dissolved in 2 ml of distilled wat The radioactivity in 0.1 ml of the dissolv hydrolyzates was measured in a Beckm LS-150 liquid scintillation counter with scintillation fluid composed of 8 g of butt PBD, 0.5 g of PBBO and 10% BBS-3 (B Solv, Beckman) per liter of toluene. some experiments, evaporated hydro zates were dissolved in 2.3 ml of distill water and assayed for proline and hydrox proline content (Switzer and Summ 1971). Occasionally the hot trichloroace acid extracts were centrifuged at 100,00 and the pellet was treated with 1.0 ml NCS solubilizer (Amersham/Searle) p 0.2 ml of insoluble suspension at 37°C at least 4 hr. The solubilized radioactiv was measured in 10 ml of scintillation flu consisting of 5 g of PPO and 0.5 g of methyl POPOP in 1 liter of toluene. T supernatant fluid (soluble collagen) th comprised about 10% of the hot trichlor cetic acid extract was acid hydrolyzed a subsequently counted.

General protein synthesis was mo tored by labeling 200 worms with [^{3}H proline, [^{3}H]leucine or [^{3}H]tryptophan 2 hr at various times during postembr onic development. After washing and ac ing carrier worms, aliquots of sonicat samples were extracted with 5% trichlor cetic acid at 95°C for 10 min. Residues we concentrated by centrifugation, wash

ith cold 5% trichloroacetic acid, metha-l and ether, dissolved in NCS or hydro-zed in 6 *N* HCl and counted in the appro-iate scintillation mixture. In other in-ances, worms that were labeled with ra-oactive amino acid were sonicated and tracted with 10% trichloroacetic acid at °C for 60 min for collagen. The soluble oline-containing pool was measured ter labeling with [^{3}H]proline for 2 hr at arious times during synchronized postem-yonic development. The labeled worms ere thoroughly washed in neutral buffer. he radioactivity present in the supernа-nt fluid after treating the sonicate with % trichloroacetic acid for 2 hr at 5°C was ssayed.

To determine the collagen content of orms at various stages of postembryonic evelopment, gram samples of synchro-ously growing nematodes were collected, ashed in 0.5 *N* acetic acid, counted by lution of 0.1-ml aliquots, sonicated and ntrifuged at 100,000*g* for 30 min (Cain nd Fairbairn, 1971). Both pellet and su-rnatant fractions were extracted with % trichloroacetic acid at 90°C for 60 min nd dialyzed against distilled water over-ight at 4°C. Protein concentration was termined (Lowry *et al.*, 1951) with urified bovine achilles tendon collagen Sigma) as the standard. After acid hydrol-sis of the collagen fraction, the protein nd hydroxyproline contents were quanti-ated (Switzer and Summer, 1971).

Tritiated protocollagen substrate, a gift om Dr. J. Koppel, for the protocollagen rolyl hydroxylase assay, was prepared om 10-day chick tibia that had been incu-ated with [3,4-^{3}H]proline and α,α-dipyri-yl (Lukens, 1966). Enzyme extracts were btained from synchronized worms that ere isolated at 4-hr intervals throughout ne L2 juvenile intermolt. Samples of 2 ml f wet packed worms were frozen at −17°C, artially thawed and ground with alumina n 5 ml of buffer [0.05 *M* KCl, 0.02 *M* ris–HCl, pH 7.2, 1% Triton X-100 (Guz-an and Cutroneo, 1973)] to which 0.05% (final volume) phenylmethylsulfonylfluo-ride (Sigma) had been added. The homoge-nate was centrifuged at 15,000*g* for 30 min. Supernatant fractions were assayed for protocollagen prolyl hydroxylase activity in the presence of 0.05 m*M* $FeSO_4$, 0.5 m*M* α-ketoglutarate, 2 m*M* ascorbic acid, cata-lase, 0.05 mg/ml, 50 m*M* Tris–HCl, pH 7.8, and about 100,000-cpm-labeled substrate at 25°C for 60 min (Hutton *et al.*, 1966). Assays were performed with extracts that had been heated to 100°C for 30 min to correct for nonenzymatically released triti-ated water.

Bacterial collagenase (Worthington, 2× recrystallized) in 0.003 *M* Tris–HCl in 0.05 *M* $CaCl_2$ at pH 6.7 was used at a substrate to enzyme ratio of 1:3 for at least 4 hr at 37°C.

RESULTS

The Time Course of Molting and Postem-bryonic Development

Figure 1 shows the time course of post-embryonic development of a population of organisms that was initially isolated dur-ing the L2 stage of maturation; that is, when the juvenile worms were about 320 ± 20 μm (±SD) in length. It is customary to partition the postembryonic period of ne-matodes into five stages. The occurrence of a molt marks the termination of one stage and the beginning of the next. Generally, under our conditions, the last three molts occur when the organisms are about 500,

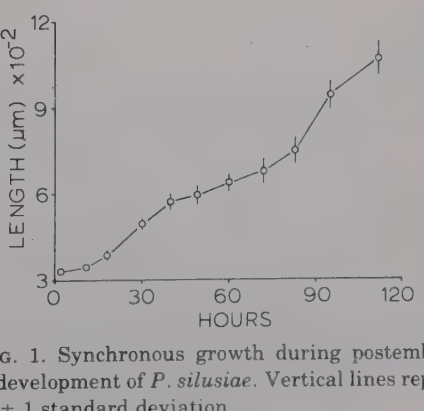

FIG. 1. Synchronous growth during postembry-onic development of *P. silusiae*. Vertical lines repre-sent ± 1 standard deviation.

650 and 850 μm in length. The adult stage commences after the fourth and final molt. The interval from the third to fourth molt, i.e., from 650 to 850 μm in length, is the fourth (L4) juvenile stage. The third juvenile stage (L3) encompasses growth from 500 to 650 μm. The L2 stage includes the size range from about 250 to 500 μm, while the period from the completion of embryonic development to the first molt is the L1 stage. An absolute rate of growth and invariant timing of each molt during postembryonic development do not occur in different experiments; however, regardless of the duration of postembryonic development during a particular experiment, the same temporal sequence of events is encountered. In many of the present experiments both growth and molting were monitored concomitantly (Westgarth-Taylor and Pasternak, 1973).

Collagen Content during Postembryonic Development

During postembryonic development there is an accelerated accumulation of hot trichloroacetic acid-extractable material per organism which amounts to an overall 50-fold increase (Fig. 2). About 60% of the total buildup of collagen occurs after the final molt. From the mid-L2 stage to the inception of the adult stage there is a 20-fold gain in the collagen content per worm. The persistent accumulation of collagen during maturation suggests that this protein(s) is being produced more or less continually. When expressed on the basis of estimated total volume per organism or relative mass per worm, the amount of collagen per mass per worm remains fairly constant up to the final molt (Fig. 2, inset).

In concert with the increasing collagen content per worm the amounts of collagen-bound proline and hydroxyproline rise (Fig. 3). The increase in collagen-bound proline is proportional to the increase in the mass (micrograms) per nematode during maturation. The ratio of hydroxyproline to proline found in collagen, regardless of the length of the worm, is 0.129 0.003, indicating that at this level of resc tion similar collagens are synthesized each stage of postembryonic developme When the values are presented as the droxyproline to proline ratio in collage a function of nematode length, a sin regression line fits the data with evide of a slight trend to an increased hydro

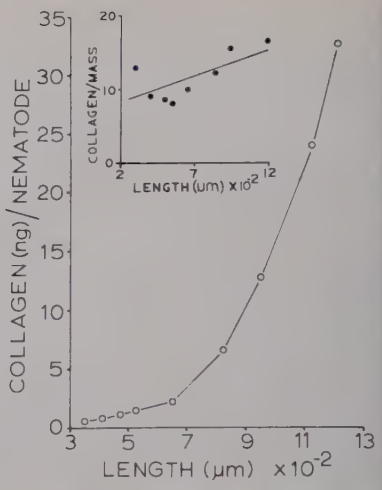

FIG. 2. Collagen content per nematode du postembryonic development. Collagen concer tion is expressed as nanograms per nematode. inset shows a plot of collagen (nanograms) per n (micrograms) as a function of worm length. T mass per worm was estimated from Andrassy's e tion as cited by Bird (1971). The coefficient cor tion of the regression line was +0.695 ($P > 0.0$ determined from the t ratio).

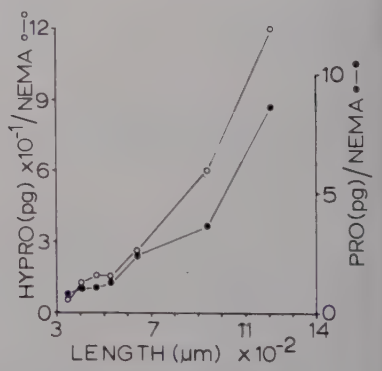

FIG. 3. Proline and hydroxyproline content collagen extracted at various times during post bryonic development. Open circles, hydroxypro closed circles, proline.

oline to proline ratio as postembryo-ic development progresses (slope = 0.0000557; $r = +0.98$).

corporation of [^{3}H]Proline during Post-embryonic Development

To measure the synthesis of collagen at arious times during postembryonic devel-pment, [^{3}H]proline was added periodi-lly for a duration of 2 hr to synchro-ously growing populations. The counts maining after extraction with hot trichlo-acetic acid were ascertained. Labeled oline was incorporated discontinuously to the collagen of developing worms dur-g the maturation period. In a representa-ve experiment shown in Fig. 4, three aks of pyrrolidine activity were ob-rved. Each peak of incorporation of label receded a molt; i.e., the time at which the d cuticle is sloughed off. In this instance, e first peak occurred when the worms ere about 380 μm in length with the ensu-g molt taking place when the worms ere 460 μm long. The third and fourth olts took place when the worms reached 0 μm and 810 μm in length, respectively. here is variation from experiment to ex-eriment in both the timing of the peaks nd the occurrence of the molts. If an ex-

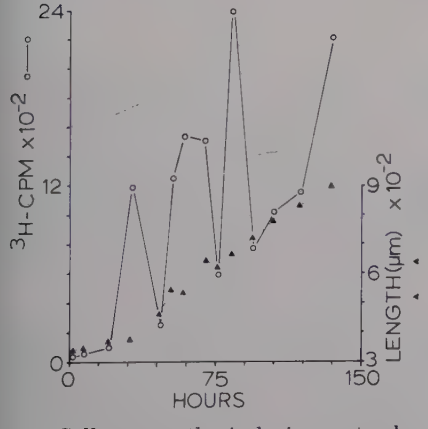

FIG. 4. Collagen synthesis during postembryonic velopment. Each open circle represents the triti-ed counts per minute extracted as collagen after 2-incubation with [^{3}H]proline. Triangles denote pat-rn of synchronous growth during this experiment.

periment is maintained into the adult stage an increase in the uptake of label follows the final molt. In these cases, eggs within the mature female have been fertilized and the offspring of the next generation have begun to develop *in utero*.

No evidence of discontinuous incorporation of [^{3}H]proline was found when the noncollagenous component; i.e., hot trichloroacetic acid-insoluble extract was examined for radioactivity. This fraction usually comprised about 10% of the total radioactive material that was present prior to extraction. Collagenase treatment had virtually no effect on the noncollagenous fraction, whereas about 81% of the radioactivity of the soluble hot acid-extracted material labeled with [^{3}H]proline during periods of either peak or nonpeak synthesis was digested.

When the rate of postembryonic development is slowed by maintaining the culture at 18°C, the [^{3}H]proline incorporation into collagen reflects the pattern observed in Fig. 4, although the time scale of the events is expanded (Fig. 5). On the other hand, when the time to reach maturity is compacted due to rapid growth, the peaks of incorporation are temporally compressed, sampling times need to be more frequent and the duration of the pulses of radioactive precursor reduced in order to observe discrete peaks of incorporation.

Studies with *Ascaris* indicated that 0.5 *M* NaCl differentially extracts cuticular collagen from muscle collagen (Fujimoto, 1968). After sonicates of *Panagrellus* that had been labeled with [^{3}H]proline for 2-hr periods at various times during postembryonic development were extracted with 0.5 *M* NaCl, dialyzed extensively, hydrolyzed and counted, three discrete peaks of activity were observed (Fig. 6). When aliquots of the nonhydrolyzed 0.5 *M* NaCl extracts were treated with 5% trichloroacetic acid at 90°C for 60 min, most of the radioactivity was recovered (Fig. 6). As well, each peak of label preceded a molt. These results indicate that the bulk (about 85%) of

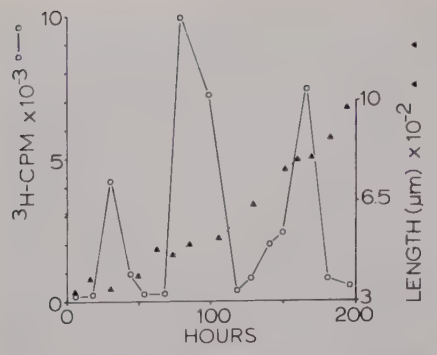

FIG. 5. Collagen synthesis at 18°C during postembryonic development. Each open circle represents the tritiated counts per minute extracted as collagen after 2-hr incubation with [^{3}H]proline. Triangles denote the pattern of synchronous growth during this experiment.

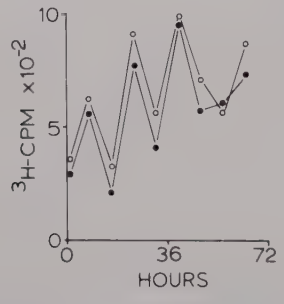

FIG. 6. Synthesis of 0.5 *M* NaCl-soluble macromolecular material during postembryonic development. Open circles represent tritiated counts per minute in dialyzed 0.5 *M* NaCl extracts after nematodes had been incubated for 2 hr with [^{3}H]proline. Closed circles signify the tritiated counts per minute found after hot trichloroacetic acid extraction of dialyzed 0.5 *M* NaCl extracts.

the 0.5 *M* NaCl-extracted material is collagenous and that muscle collagen, if the conditions that apply to *Ascaris* muscle collagen obtain here, does not contribute appreciably to the total of the hot trichloroacetic acid-extracted material.

In general, nonrandom fluctuations in the amino acid pool during postembryonic development may distort the results of pulse-labeling experiments. In our case, the relative amount of label incorporated into the overall trichloroacetic acid-soluble pool remains constant during maturation whereas discrete oscillations of incorporation into hot acid extracts were observed in split samples (Table 1). Additional experiments indicated that when L2 and adult worms were pulsed with [^{3}H]proline for 2 hr and then chased for 3 hr with unlabeled precursor there was a 9 and 5% increase, respectively, in the radioactive collagen. The latter data suggest that 2 hr is sufficient time for the equilibration of the internal proline-containing pool. At present, it is not feasible to determine either the extent of amino acid compartmentalization in *Panagrellus* or the behaviour of the hypodermal amino acid pool directly. These limitations preclude an accurate determination of the specific activity of proline in the acid-soluble pool(s) during postembryonic development.

Labeled Pyrrolidine Content of Collagen Synthesized during Postembryonic Development

The data in Table 2 show that the amount of polypeptide-bound [^{3}H]pyrrolidine in hot trichloroacetic acid extract oscillated during postembryonic development. Again, each period of relative elevated radioactivity preceded a molt. The ratios of labeled hydroxyproline to proline in this experiment had a mean value of 0.126 ± 0.019 with a meagre tread to increased hydroxyproline content in e

TABLE 1

ACID EXTRACTS AFTER 2-HR EQUILIBRATION WITH [^{3}H]PROLINE AT VARIOUS TIMES DURING POSTEMBRYONIC DEVELOPMENT

Period of labeling after synchronization (hr)	Trichloroacetic acid-soluble fraction (cpm/200 worms)	Hot trichloroacetic acid extract (cpm/200 worms)
8–10	2280	1500
10–12[a]	2355	3000
22–24	1995	1155
34–36[a]	2190	2520
46–48	2295	1305
58–60[a]	2520	2880

[a] Peak periods of collagen synthesis.

TABLE 2

[^{3}H]PYRROLIDINE CONTENT OF COLLAGEN SYNTHESIZED AT VARIOUS TIMES DURING POSTEMBRYONIC DEVELOPMENT

eriod of abeling ter synhroniza- ion (hr)	Length (μm)	Activity in collagen (cpm/200 nematodes)	
		Proline	Hydroxyproline
2–4	328.3 ± 7.2	291	18
7–9	381.7 ± 9.3	1397	141
19–21	406.1 ± 13.3	445	57
24–26	423.0 ± 10.8	665	93
31–33	487.1 ± 14.0	1656	181
44–46	592.5 ± 22.4	458	68
54–56	707.4 ± 21.7	1954	236
65–67	1004.7 ± 50.2	785	117

acted collagen as postembryonic develop- ent proceeds (slope of the regression line +0.0000695; $r = +0.64$). An overall ratio labeled hydroxyproline to proline of 113 ± 0.04 in extracted collagen was ob- ined from a number of samples ($N = 36$) worms that had been labeled at various mes during maturation.

rotocollagen Prolyl Hydroxylase Activity during Postembryonic Development

Extracts of worms that had been iso- ted at successive times during the L2 ntermolt period were assayed for protocol- gen prolyl hydroxylase activity. The re- lts show that the enzymatic activity in- eases to a peak and then decreases prior the second molt (Table 3). This pattern protocollagen prolyl hydroxylase activ- y parallels collagen synthesis. Similar ualitative hydroxylase activity profiles ere observed during the L3 and L4 inter- nolts (not shown).

ncorporation of [^{3}H]Tryptophan and [^{3}H]Leucine during Postembryonic Development

To determine whether the pattern of H]proline incorporation into collagen dur- ng postembryonic development was com- ensurate with periodic bursts of general protein synthesis, tryptophan, an amino acid not found in the collagen of *Ascaris* and other invertebrates (Kirschenbaum, 1973), was used as a polypeptide precursor. Samples of synchronously growing nematodes at various times during postembryonic development were collected and subdivided. One group was incubated with [^{3}H]proline and the other with [^{3}H]-tryptophan, each for 2 hr. Subsequently, each sample was split and processed for either noncollagenous protein or collagen. Specific peaks of radioactive protein were not present in either the noncollagen (Fig. 7A) or collagen (not shown) fractions after the administration of [^{3}H]tryptophan during postembryonic development, while the pattern of discontinuous incorporation of [^{3}H]proline into nondialyzable hot trichloroacetic acid-soluble extracts was apparent (Fig. 7A). In contrast with the repeatability of the pattern of [^{3}H]proline incorporation into collagen during postembryonic development, the profile of the uptake of [^{3}H]tryptophan into noncollagenous proteins, i.e., hot acid-insoluble material, was variable from one experiment to the next.

In other experiments, [^{3}H]leucine, a minor component of invertebrate collagens (Kirschenbaum, 1973), was used to monitor protein synthesis during postembryonic development in the usual manner. In

TABLE 3

PROTOCOLLAGEN PROLYL HYDROXYLASE ACTIVITY DURING THE L2 INTERMOLT

Nematode length (μm)	Protocollagen prolyl hydroxylase activity[a] (cpm)
390	10,000
444	48,000
485	70,000
540	40,000

[a] Activity is expressed as counts per minute of [^{3}H]water released from an extract of 2 ml of wet packed nematodes after correcting for $[^3H]_2O$ release from extracts that had been heated to 100°C for 30 min. These data represent one experiment. Similar qualitative results were observed in other experiments.

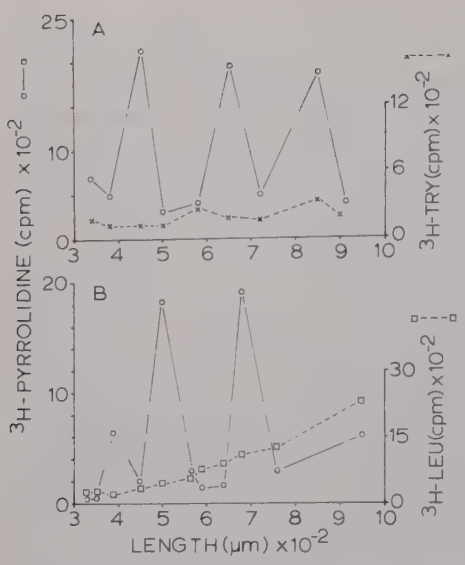

FIG. 7. Collagen and protein synthesis during postembryonic development. (A), Split samples were labeled with either [^{3}H]tryptophan (×) or [^{3}H]-proline (○) for 2-hr intervals during postembryonic development and extracted for noncollagenous protein (×) and collagen (○). (B), One series of samples was labeled with [^{3}H]leucine for 2-hr intervals during postembryonic development and extracted for noncollagenous proteins (□); the other series was labeled with [^{3}H]proline and extracted for collagen (○).

this case, the relative rate of incorporation of labeled leucine into noncollagenous proteins (hot acid-insoluble material) increased with increasing nematode size (Fig. 7B). There were no obvious signs of discontinuous production of noncollagenous proteins that contain leucine during postembryonic development.

Pulse and Chase Experiments

The protocol of the pulse–chase experiments was to incubate synchronously growing worms with [^{3}H]proline for 2-hr intervals during postembryonic development. Each sample was split. One set was processed in the usual manner for collagen at the end of each incubation period while the other was washed, cultured in the presence of unlabeled proline and subsequently assayed for collagen at 10, 26 and 36 hr after the initial labeling period. The pattern of retention of label in hot trich roacetic acid-extractable material from representative experiment is shown in T ble 4. In this example, the peaks of incor ration of proline into collagen occurred about 22, 38 and 62 hr after synchroni tion. After 10 hr of chase, there was overall 18.2% mean loss of labeled collag from the previously labeled samples. By hr, most of the pulsed samples had pass through an ecdysis. In the sets of org nisms that were labeled preceding a pe of collagen production (Table 4, rows 1 and 5) a moderate loss of label had curred (mean loss = 19.6%), whereas sa ples labeled at peak periods (Table 4, ro 2, 4 and 6) lost about 71.5% (mean value) their original material. By 36 hr, sampl that had been labeled at peak periods h undergone a mean loss of about 82.1% the original radioactivity, while the se that were labeled during nonpeak tim showed a retention of 78.4% (mean) of t original label. Similar results were c tained in four additional experiments.

DISCUSSION

In the present study we have demc strated that collagen biosynthesis duri postembryonic development in the free-li ing nematode *Panagrellus silusiae* is d continuous. Each increase in uptake of pr

TABLE 4

FATE OF COLLAGEN SYNTHESIZED AT VARIOUS TIM DURING POSTEMBRYONIC DEVELOPMENT

Period of labeling after synchronization (hr)	Activity in collagen after labeling period (cpm per 200 nematodes)			
	0 Hr	10 Hr	26 Hr	36 Hr
14–16	269	238	197	180
22–24[a]	485	318	170	100
30–32	287	294	246	230
38–40[a]	614	559	161	98
52–54	139	164	116	135
62–64[a]	434	227	106	N.A.[b]
74–76	208	194	N.A.[b]	N.A.[b]

[a] Period of peak collagen synthesis.
[b] Not analyzed. L1 juveniles present in culture

line into polypeptide material preceded a molt. Moreover, the observed pattern of collagen synthesis was unique in that there was no evidence, at the present level of resolution, of coincident bursts of general protein synthesis. Since the cyclic variations of proline incorporation were not coupled to parallel fluctuations in the size of the proline-containing amino acid pool, the pattern of collagen production appears to be due to a differential rate of synthesis. However, it should be emphasized here that the endogenous production of proline during postembryonic development was not measured directly by our experiments and therefore the observed alterations in collagen biosynthesis may be due to regulated changes in the specific activity of the intracellular proline pool. In this species the old cuticle is not resorbed during ecdysis (Samoiloff and Pasternak, 1969); thus, the periods of reduced proline incorporation into collagen are not due to a buildup of amino acid from that particular source. In addition, it remains to be determined whether the elevations of collagen synthesis during the last three intermolts are due to the production of cuticular collagen per se. Nonetheless, as a working hypothesis, we postulate that the observed pattern represents a temporally modulated synthesis of the collagenous components of the cuticle during postembryonic development. At present, support for this premise is inferential but compelling when considered together with other data from the literature. Some of the elements for this interpretation include: 1) Each peak of elevated proline incorporation into collagen comes before each molt; 2) each peak of proline uptake coincides with signs of extensive biosynthetic activity in the tissue that produces the new cuticle (Samoiloff and Pasternak, 1969; Bonner and Weinstein, 1972); 3) the cuticle contains a large quantity of collagenous protein (Lee, 1966; Bird, 1971); 4) the cuticle comprises a substantial component of the mass of the worm; 5) in *Ascaris*, protocollagen prolyl hydroxylase activity rises initially prior to the first molt (Cain and Fairbairn, 1971); 6) as observed here, there is a substantial loss after ecdysis of putative cuticular collagen that was labeled at a peak time; in *Nippostrongylus brasiliensis* similar pulse and chase experiments showed that about 35% of the label present in an insoluble form after administration of labeled proline during the L3 stage was found subsequently associated with the sloughed off cuticle (Bonner *et al.*, 1971); and 7) the amino acid compositions of hot acid-soluble extracts isolated at peak synthetic times and partially purified cuticular fragments of *Panagrellus* are identical (unpublished data). Both the relative constancy of the hydroxyproline to proline ratios and the nonzero levels of collagen production during the three intermolt periods suggest that the same kind of collagen is being synthesized continuously throughout maturation, which, in turn, implies that the increased synthesis prior to each molt is due to quantitative regulation.

We cannot account for the persistence through successive ecdyses of collagen that was synthesized during nonmolting periods if that collagen is, indeed, cuticular in nature. Such a pattern of retention may be related to the mode of processing and secretion of cuticular components that are produced at specific times during postembryonic development. In general, cuticle production has two aspects. First, there is a continual formation of cuticular elements that are added to an existing cuticle throughout maturation. Second, there is a period of intense synthetic activity followed by the assembly of an intact new cuticle prior to each ecdysis. It is possible that much of the cuticular collagen produced during the intermolt is not readily mobilized into the cuticle, becomes trapped within the hypodermis and eventually loses the ability to become incorporated into the cuticle. On the other hand, much of the cuticular collagen synthesized at the time of a peak is probably promptly

assembled into a new cuticle. Subsequently, a large relative proportion of this material is lost when the cuticle is sloughed off at the ecdysis.

The hydroxyproline to proline ratio observed in hot trichloroacetic acid extracts from *Panagrellus* is about twice the observed value in purified cuticular collagen isolated from *Ascaris* (McBride and Harrington, 1967; Fujimoto and Kanaya, 1973). This difference may reflect the presence of a distinctive cuticular collagen or a combination of collagens in *Panagrellus*. Of course, the occurrence of adventitious noncuticular collagens in the hot acid-soluble extracts cannot be excluded. Part of the variation could, also, be due to the extent of hydroxylation that occurs naturally in the two forms. In *Ascaris* the level of hydroxylation of cuticular collagen can be manipulated experimentally by raising the relative O_2 tension in the surrounding medium (Chvapil and Ehrlich, 1970). Under natural conditions adult *Ascaris* exists in a low O_2 environment, and the level of hydroxyproline in cuticular collagen is low while hydroxylysine cannot even be scored. By contrast, free-living nematodes are both found and maintained at high O_2 levels. Therefore, one might expect higher cuticular collagen hydroxyproline and hydroxylysine levels in these forms than in *Ascaris*. In fact, collagen-bound hydroxylysine has been found in extracts of cuticular fragments of *Panagrellus* (unpublished data).

The significance of the hydroxyproline to proline ratio data is problematic. After culturing *N. brasiliensis* in 95% air:5% CO_2, the ratio of labeled hydroxproline to proline in the cuticle released after ecdysis was about 0.76 (Bonner *et al.*, 1971). If it is assumed that there was no selective loss of a major proline-rich cuticular component, then this ratio is about six times the value observed in *Panagrellus* collagen extracts. Perhaps the cuticular collagens of different nematodes are divergent.

Protocollagen prolyl hydroxylase activity is present in developing eggs (Cain and Fairbairn, 1971) and both adult muscle and hypodermis (Fujimoto and Prockop, 1969; Chvapil *et al.*, 1970) of *Ascaris*. During embryogenesis in *Ascaris* two successive peaks of protocollagen prolyl hydroxylase activity occur. The first peak coincides with the first molt, while the second one has no definite structural correlate. In other systems, a stimulation of collagen production is accompanied by increased protocollagen prolyl hydroxylase activity (Takeuchi *et al.*, 1967; Langner and Fuller, 1969). In *Panagrellus*, elevation of protocollagen prolyl hydroxylase activity precedes each molt and reflects the pattern of collagen production. Regardless of whether this enzyme is specifically activated (Stassen *et al.*, 1973) or induced *de novo*, our findings indicate that during each molting cycle a coordinately controlled set of processes is manifested.

The level at which the macromolecular information that is required for the molting cycle is regulated has not been clarified. The administration of actinomycin D during the second intermolt of *N. brasiliensis* effectively blocked the organization of the L3 cuticle (Bolla *et al.*, 1972). As well, previous work with *P. silusiae* indicated that each molting cycle was dependent upon continued RNA and/or protein synthesis during the intermolt (Westergarth-Taylor and Pasternak, 1973). In experiments to be published we have shown that both transcription and translation during each intermolt are required for post-embryonic collagen synthesis whereas growth per se is not a necessary condition for the maintenance of the temporal pattern of collagen synthesis.

Previously, neurosecretory activity has been implicated as an overall regulator of molting in nematodes (Davey, 1966). Recently, independent sites for the control of molting and ecdysis in *Panagrellus* have been deduced from laser beam lesion studi

es (Samoiloff, 1973). As yet, the basis for he oscillatory nature of cuticle production during postembryonic development is not known. In sum, the present data suggest that a programmed schedule of events functioning at different levels of organization is repeated within each intermolt period during the postembryonic development of a nemotode.

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Gene Activation during Muscle Differentiation and the Role of Nonhistone Chromosomal Protein Phosphorylation

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The phosphorylation of nuclear proteins, in a standard assay with nuclei isolated from chick muscle cell cultures, increases steadily with time in culture. This increase is shown to reflect increased nuclear protein kinase activity. Most of the phosphorylated protein is chromatin bound and so are the enzymes responsible for the phosphorylation. Analysis of chromosomal proteins on hydroxylapatite columns shows that ^{32}P is incorporated almost entirely into nonhistone proteins, while the specific radioactivity of the histones is low and does not increase during cell culture.

Experiments in which cells were grown at different initial densities and, consequently, underwent cell fusion at different times show that phosphorylation is dependent neither on cell density nor on the degree of cell fusion. Chick thigh fibroblasts, prepared by repeated subculture of fibroblasts from muscle cell preparations to eliminate myogenic cells, do not show the increase in phosphorylation with culture time, suggesting that the changes may be specific for primary myoblast cultures. These changes are not shown by thigh muscle nuclei isolated during development in the whole embryo. In fact, nuclear protein phosphorylation activity decreases rapidly *in ovo* with increasing embryonic age.

Possible roles for phosphorylation of nonhistone chromosomal proteins in gene activation during myogenesis are discussed in the light of these observations.

INTRODUCTION

The phosphorylation of nonhistone chromosomal (NHC) proteins has been implicated in gene activation and specific changes in the pattern of gene transcription (Stein *et al.*, 1974; Allfrey *et al.*, 1973). Since dramatic changes in the patterns of RNA and protein synthesis occur during muscle differentiation *in vitro* (Morris *et al.*, 1972; Paterson and Strohman, 1972; Heywood *et al.*, 1973; Nguyen thi Man and Cole, 1972, 1974), we have begun an investigation of the relationship of NHC protein phosphorylation to these changes.

In the first 4 days of culture *in vitro*, chick thigh muscle cells divide rapidly, fuse to form multinucleate myotubes and begin to accumulate contractile proteins characteristic of mature muscle (Fischman, 1970). In an earlier publication (Nguyen thi Man *et al.*, 1974), we showed that the rate of protein phosphorylation in isolated nuclei increases steadily during this culture period. The phosphorylation assay with isolated nuclei essentially measures the activity of nuclear protein kinases using endogenous nuclear protein as substrate.

Changes of this kind have been observed in other differentiating systems. Thus, stimulation of lymphocytes by phytohemagglutinin (Kleinsmith *et al.*, 1966) and mammary cells by prolactin (Turkington and Riddle, 1969), both associated with gene activation, are associated with increased phosphorylation activity, whereas red blood cell maturation, involving increasing restriction of genome expression, is accompanied by a decrease in NHC protein phosphorylation (Gershey and Kleinsmith, 1969). NHC protein phosphorylation and chromatin-bound protein kinase activity are both increased in folic-acid-induced kidney regeneration (Brade *et al.*, 1974). In these cases, however, there is also a correlation with cell division, since

mammary cell and lymphocyte differentiation and kidney regeneration involve increased cell proliferation, while cell proliferation ceases as red blood cells mature. This correlation does not exist in myogenesis *in vitro,* however, since NHC protein phosphorylation continues to increase even as cells fuse and stop dividing.

Changes in the nature or degree of phosphorylation of NHC proteins might influence the pattern of gene transcription either by directly affecting an interaction between NHC proteins and DNA or by affecting the interaction between NHC proteins and the nucleohistone complex (Kleinsmith *et al.,* 1966; Paul and Gilmour, 1968; Johnson *et al.,* 1973). Kamiyama *et al.* (1972) have shown that the ability of nonhistone phosphoproteins to stimulate RNA synthesis from nucleohistone increases with increasing phosphate content, whereas Shea and Kleinsmith (1973) have made a similar observation using DNA alone. The latter workers, using homologous mammalian RNA polymerase, showed that the stimulation was specific for homologous DNA. Clearly a mechanism for specificity is required at some stage for an increase in protein kinase activity to give rise to new patterns of transcription, but if such a mechanism preexisted in the types and arrangement of nuclear proteins and DNA in a muscle precursor cell, then a general increase in the degree of phosphorylation of these proteins might act as a trigger to realise this potential.

With these considerations in mind we have tried to devise experiments to determine what kinds of nuclear protein are phosphorylated in nuclei from cultured chick muscle cells and whether the phosphorylation is related to gene activation or to some other process occurring in the cultures. The present studies show that the increase in phosphorylation activity during cell culture is due to increased phosphorylation of chromatin-bound nonhistone proteins by kinases that are also mainly chromatin bound. We rule out the possibility that the increase in phosphorylation activity is tightly coupled to either the increasing cell density or the increasing degree of cell fusion in the cultures. Our data encourage the idea that this increase is specific for primary muscle cell cultures, since chick thigh fibroblasts (prepared by selection and repeated subculture of muscle-derived cells) do not show an increase in nuclear phosphorylation activity with time in culture.

However, when nuclei were isolated directly from embryonic thigh muscle during the extensive period of cell fusion and myogenesis *in ovo,* nuclear protein phosphorylation activity was found to decrease dramatically with increasing embryonic age and this observation would seem to argue against a necessary relationship between increased protein kinase activity and muscle differentiation.

The possibility that NHC protein phosphorylation is an important step in myogenesis is examined in the light of these observations, and alternative explanations for the increase in nuclear protein kinase activity are considered.

MATERIALS AND METHODS

Materials. All materials used for cell culture were purchased from Flow or Bio-Cult laboratories. [γ-^{32}P]ATP (ammonium salt, 16–19 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. An 8 *M* urea stock solution was prepared from Analar grade urea (B.D.H. Poole, Dorset, U.K.) and then filtered and deionized by passage through a column of AG 501-X8 (D) mixed bed resin (Bio-Rad Laboratories Ltd., St. Albans, Hertfordshire, U.K.). Hydroxylapatite (Bio-Gel HT) was obtained from Bio-Rad Laboratories, and casein (Hammarsten) from B.D.H. Ltd.

Cell culture. Cells were prepared from thigh muscles of 12-day chick embryos by a modification of the mechanical method of Tepperman *et al.* (manuscript submitted)

as described previously (Nguyen thi Man and Cole, 1974). In all experiments, except as otherwise stated, cells were plated at an initial density of 15×10^6/135-mm collagen coated petri dish in 20 ml of Ham's F-10, supplemented with 10% selected horse serum, 2% chick embryo extract, 1 m*M* glutamine, penicillin (50 U/ml), and streptomycin (50 μg/ml). Cultures were fed daily, except on the 2nd day, with a complete change of medium.

Before plating, all cell preparations from thigh muscle of 12-day chick embryos were preselected for 30 min on a glass surface to enrich the myoblast population. Muscle-derived fibroblast cultures were prepared from the fibroblasts contaminated with myoblasts which attached to the substratum. These were then grown for 3–4 days in the same medium as the myoblast cultures, during which time many of the remaining myoblasts in the mixed population fused. Cells were then trypsinized, filtered through one layer of nylon gauze, centrifuged at 500*g* for 1 min and replated at 15×10^6 cells/plate. After three successive passages, muscle fibres no longer developed in the cultures and all cells were of flattened fibroblastic appearance. They were then grown and harvested for isolation of nuclei under exactly the same conditions as for myoblast cultures and grew at similar rates.

Cell fusion was determined after fixing and staining replicate plates, as described previously (Morris and Cole, 1972). After selection, muscle cell cultures prepared by the present method contained 16% fibroblasts as judged by morphological criteria (Morris and Cole, 1972), and this proportion did not increase during the first 4 days of culture. Sixty to seventy percent of the remaining myoblasts fused by the 4th day.

Isolation of nuclei. All cultures were fed with a complete change of medium 2 hr before harvesting. They were then rinsed with cold saline G (Puck *et al.*, 1958), harvested with a rubber policeman and collected by centrifugation. Nuclei were isolated as described previously (Nguyen thi Man *et al.*, 1974), and a slight modification of this method was used to isolate nuclei from embryonic muscle tissue.

In experiments where cells were prepared from embryonic muscle before preparation of nuclei, muscle preparations of different embryonic ages were treated identically, filtered through nylon and preselected on glass surfaces before harvesting by centrifugation.

Cell pellets (about 0.1 ml of packed cells or tissue) were resuspended in 7 ml of RSB (0.01 *M* NaCl, 0.0015 *M* $MgCl_2$, 0.01 *M* Tris–HCl, pH 7.4) containing 1% Triton X-100 and homogenised by hand with seven strokes of a tight-fitting Dounce homogeniser. After leaving for 10 min at 0°C, the homogenisation was repeated and the homogenate was centrifuged at 500*g* for 10 min. Tissue homogenates were filtered through nylon gauze before centrifugation. The nuclear pellet was then resuspended in RSB without Triton X-100 and the homogenisation and centrifugation steps were repeated. All operations were performed at 0–4°C, and nuclei were stored at −40°C.

Nuclei were quantitated by their absorbance at 260 nm, 1 A_{260} unit of nuclei containing 155 ± 5 μg of protein estimated by the method of Lowry *et al.* (1951). This value and the DNA to protein ratio (1:4.3) do not change during the first 4 days of culture, and the same is true for embryonic nuclei over the period studied here.

Quantitative assay of phosphorylation of nuclear proteins. The incorporation of ^{32}P from [γ-^{32}P]ATP into total nuclear proteins was determined by the method of Rickwood *et al.* (1973) as described in detail earlier (Nguyen Thi Man *et al.*, 1974). All experiments were performed in duplicate or triplicate and zero-time background incorporation was subtracted from all results.

Qualitative analysis of nuclear phosphoproteins. The method described below derives essentially from that of MacGillivray

et al. (1972). Nuclei (6–7 A_{260} units) were incubated at 37°C in 0.25 *M* sucrose–0.01 *M* $MgCl_2$–0.025 *M* NaCl–0.1 *M* Tris–HCl, pH 8.0 (known as phosphorylation buffer) and 50 μCi of [γ-^{32}P]ATP (specific radioactivity, 16–19 Ci/mmol) in a total volume of 3 ml for 5 min. The reaction was stopped by quickly cooling the samples in wet ice and by centrifugation at 1000*g* for 10 min at 4°C. The nuclear pellet was washed three times with 5 ml of 2.5 m*M* cold ATP in phosphorylation buffer to remove free phosphate. The nuclear pellet was then extracted with 50 volumes of 0.14 *M* NaCl–0.05 *M* Tris–HCl, pH 7.5, by homogenization with a Teflon Dounce-homogenizer for 30 sec and centrifuged at 15,000*g* for 15 min. The extraction was repeated once more.

Chromatin solutions were then prepared by homogenizing the nuclear pellet in 50 volumes of 5 *M* urea–2 *M* NaCl–1 m*M* Na phosphate, pH 6.8, using a Teflon Dounce-homogenizer. After centrifugation at 15,000*g* for 20 min, the pellet was extracted once more. The two chromatin solutions were combined and sonicated twice, each time for 15 sec, using an MSE ultrasonic power unit at 2 A with 1-min cooling in iced water. Chromatin solutions were then ready to be chromatographed on hydroxylapatite column. By this method 50–70% of the protein was extracted from chromatin, and the insoluble residue, analysed by gel electrophoresis, resembled the extractable NHC proteins (unpublished).

Hydroxylapatite (Bio-Gel HT) was washed several times with 5 *M* urea–2 *M* NaCl–1 m*M* Na phosphate, pH 6.8. Chromatin samples, 10–12 ml, in 5 *M* urea–2 *M* NaCl–1 m*M* Na phosphate, pH 6.8, were applied to a 3 × 2-cm column of hydroxylapatite and allowed to run at room temperature. Histones were not retained by the column and were eluted with 1 m*M* Na phosphate. The nuclear proteins retained on hydroxylapatite were eluted stepwise with 0.05, 0.2 and 0.5 *M* Na phosphate, pH 6.8, in 5 *M* urea–2 *M* NaCl. These proteins have an excess of acidic amino acids over basic and were therefore considered acidic or nonhistone proteins (MacGillivray *et al.*, 1972). Recoveries from the column of between 50% and over 70% are consistent with the observations of MacGillivray *et al.* (1972).

RESULTS

Figure 1 shows early increases in both cell fusion and protein phosphorylation activity of isolated nuclei from chick thigh muscle primary cell cultures. Phosphorylation continues to increase at least until the 5th day while cell fusion reaches a plateau value of 50–60% on the 3rd or 4th day (Nguyen thi Man *et al.*, 1974). We have previously shown that the increased ^{32}P incorporation reflects an increase in the initial rate of protein phosphorylation and is not an artifact due to a decrease in nuclear ATP pools (Nguyen thi Man, *et al.*, 1974).

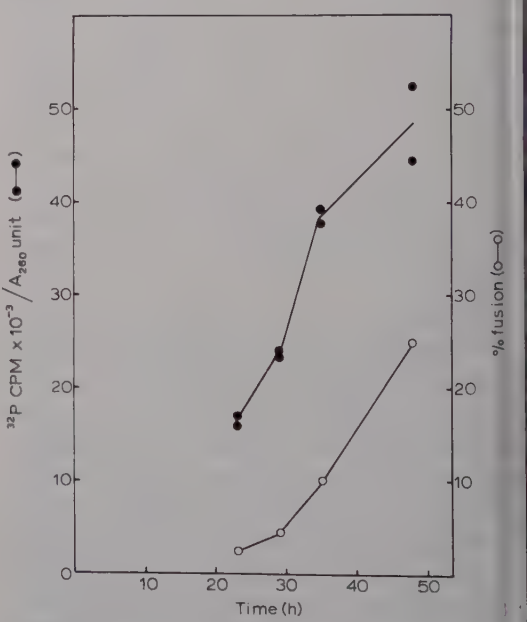

FIG. 1. Quantitative changes in nuclear protein phosphorylation activity during muscle differentiation *in vitro*. The incorporation of ^{32}P into protein of nuclei isolated at different times of cell culture (●——●) is shown in relation to cell fusion (○——○). Each duplicate incubation contained 0.9 A_{260} unit of nuclei.

Since the phosphorylation assay involves intact nuclei, changes in ^{32}P incorporation could in theory be due either to changes in the activity of nuclear protein kinases or phosphatases or to changes in the concentration or accessibility of the endogenous protein substrates. To examine these possibilities we prepared a partially purified kinase from the nuclei at different stages of culture by phosphocellulose chromatography (Takeda *et al.*, 1971) as shown in Fig. 2. As described by Ruddon and Anderson (1972), several tiny but distinct peaks of kinase activity were eluted before the main peak, but these made no significant contribution to the total activity.

Kinase activity derived from cells isolated at different times in culture was assayed in the presence of 300 μg of casein substrate, while less than 5 μg of nuclear protein were contributed by the enzyme fraction. The results in Table 1 show that increased protein kinase activity is at least partially responsible for the increase in ^{32}P incorporation by isolated nuclei, but the fact that a greater percentage increase is shown with endogenous substrate (unfractionated nuclear proteins) may mean that there is also an increase in the concentration of effective substrate sites (serine and threonine side chains) relative to the total absorbance at 280 nm.

The possibility that a decreasing activity of a protein phosphatase plays a role in the increase of phosphorylation is discouraged by the evidence of Fig. 3, which shows a time course of ^{32}P incorporation in nuclei from 24-hr cultures. The incorporation stops after 15–20 min, and the stimulation on adding more [γ-^{32}P]ATP after 10 min shows that this is due to breakdown of ATP by ATPases in the nuclear preparation. Our measurements of liberation of ^{32}P from [γ-^{32}P]ATP as free phosphate during the incubation by the method of Rickwood *et al.* (1973) are consistent with this.

Addition of a large excess of unlabeled ATP after 10 min reveals a low rate of dephosphorylation, which may be energy dependent, since, in the absence of added unlabeled ATP, a plateau level of ^{32}P incorporation is maintained. Kleinsmith *et al.* (1966) have shown that protein dephosphorylation is coupled to an energy-dependent reaction in calf thymus nuclei. This being so, the rate of dephosphorylation will be much lower during the initial 10-min label-

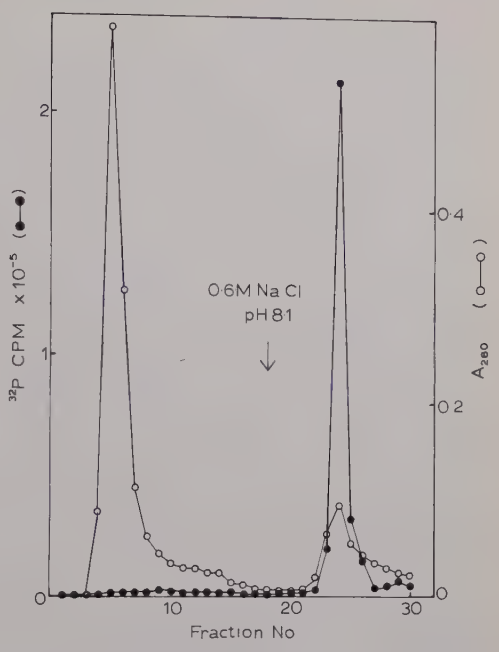

FIG. 2. Phosphocellulose chromatography of nuclear proteins and assay of protein kinase activity. Nuclei were prepared directly from 12-day embryonic thigh muscle and resuspended in 5 ml of 1 *M* NaCl–0.05 *M* Tris–HCl, pH 7.4 (Takeda *et al.*, 1971). After sonication, the suspension was centrifuged at 25,000*g* for 30 min and the pellet was extracted once more in the same way. The pooled supernatant fractions were dialysed overnight against 0.4 *M* NaCl–0.05 *M* Tris–HCl, pH 7.4 and centrifuged once more before they were applied to a 6 × 2-cm column of phosphocellulose (Whatman P-11). Five-milliliter fractions were collected, and the arrow indicates the point at which the buffer was changed to 0.6 *M* NaCl–0.05 *M* Tris–HCl, pH 8.1. Aliquots, 0.5 ml, were assayed for protein kinase in a total of 1 ml of the following solution (final concentrations): [γ-^{32}P]ATP, 5 μCi; casein, 300 μg; NaCl, 0.3 *M*; $MgCl_2$, 0.01 *M*; Tris–HCl, pH 8.0, 0.05 *M*. Protein was precipitated, washed with 0.75 *M* perchloric acid and dissolved in a Triton scintillation fluid for determination of radioactivity. All operations were performed at 0–4°C.

TABLE 1

CHANGES IN PROTEIN KINASE ACTIVITY OF DISSOCIATED CHROMATIN AND A PARTIALLY PURIFIED ENZYME FRACTION DURING CELL CULTURE[a]

Age of culture (hr)	^{32}P incorporated (cpm/A_{280} unit of nuclear extract applied to the column)	
	Before chromatography, no added casein	Second peak of Fig. 2 + 300 μg of casein
24	2,780 ± 1,080 (2)	10,540 ± 2,690 (3)
72	13,145 ± 2,810 (3)	21,520 ± 3,860 (2)
144	21,848 ± 2,710 (3)	29,330 ± 4,180 (2)

[a] Extracts of nuclei were prepared for phosphocellulose chromatography as described in the legend to Fig. 2. After dialysis and centrifugation, the A_{280} was determined and 3 A_{280} units of each nuclear extract were applied to three identical columns. After increasing the salt concentration to 0.6 *M*, the three 5-ml fractions from each column containing uv-absorbing material were pooled and 0.5-ml aliquots were assayed, as described in the legend to Fig. 2, together with aliquots of the original nuclear extract. Values are shown ± standard errors of the means and are expressed in terms of the A_{280} loaded onto the column rather than the A_{280} eluted by 0.6 *M* NaCl. Numbers in parentheses indicate numbers of experiments used to obtain average values.

ing since the concentration of ATP is so much lower. This is confirmed by the observation that a further aliquot of [γ-^{32}P]ATP produced a further burst of incorporation equivalent to the initial one rather than a stimulation of phosphatase activity to remove the ^{32}P already incorporated. Furthermore, the phosphatase activity would have to be highest in 24-hr nuclei if a decrease in the activity is to make a significant contribution to the overall increase in ^{32}P incorporation during development *in vitro*.

We next approached the problem of what kinds of nuclear protein are phosphorylated in the *in vitro* assay. After incubation of nuclei with [γ-^{32}P]ATP, chromatin was prepared by two successive homogenizations in isotonic saline (MacGillivray *et al.*, 1972) and the incorporation into both chromatin and saline-soluble proteins was estimated. Figure 4 shows the increase in incorporation in both these fractions with increasing cell culture time. Phosphorylation of both fractions shows a similar percentage increase but most (about 95%) of the labeled protein is associated with the chromatin fraction.

In order to investigate the distribution of the protein kinase between chromatin and saline-soluble fractions, nuclei were fractionated and then incubated separately with [γ-^{32}P]ATP in the presence of casein as substrate. The results show that about two-thirds of the total enzyme activity is associated with the chromatin (Table 2), suggesting either that a significant amount of the kinase is free in the nucleoplasm of the living cell or that the enzyme is more easily removed from the chromatin by the fractionation procedure than are its chromosomal protein substrates. Neither fraction is significantly affected by cyclic AMP. The low incorporation in the saline-soluble fraction without added casein is probably due to the low concentration of protein substrate.

Chromatin was dissociated with a high salt–urea buffer and the chromosomal pro-

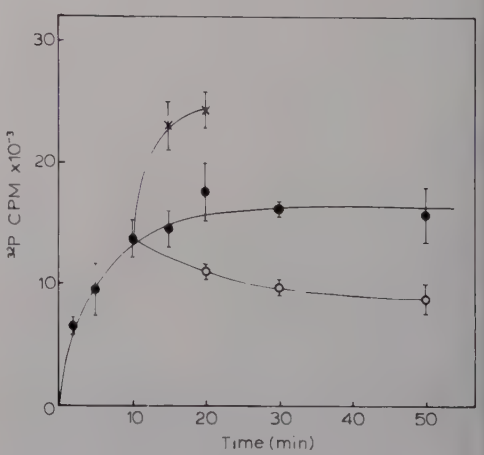

FIG. 3. Time course of ^{32}P incorporation into nuclear protein (●——●) and the effect of adding, after 10-min incubation, unlabeled ATP (50-fold excess, ○——○) or additional [γ-^{32}P]ATP (5 μCi, as initially, ×——×). Vertical bars show the standard errors of the means of triplicate incubations, and each incubation contained 0.47 A_{260} unit of nuclei from 24-hr cultures.

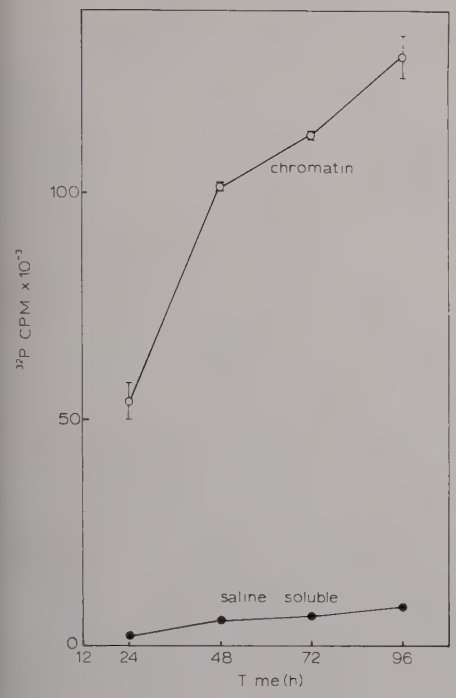

FIG. 4. Incorporation of ^{32}P into chromatin (O——O) and saline-soluble proteins (●——●) of nuclei isolated at different times of cell culture. Each incubation contained 7.2 A_{260} units of nuclei and was labeled with 30 μCi for 5 min at 37°C in 3 ml of phosphorylation buffer (0.25 *M* sucrose–0.1 *M* Tris–HCl (pH 8.0)–10 m*M* $MgCl_2$–25 m*M* NaCl). The incubation was terminated by quickly cooling the tubes in iced water followed by centrifugation at 1000*g* for 10 min at 4°C. The pellet was then washed three times with 50 volumes of 2.5 m*M* cold ATP in phosphorylation buffer, followed by homogenisation in 50 volumes of 0.14 *M* NaCl–50 m*M* Tris–HCl, pH 7.5, for 30 sec and by centrifugation at 15,000*g* for 15 min to remove saline-soluble proteins. This extraction was repeated and the two supernatant fluids were combined. The saline-soluble and chromatin fractions were made 10% in trichloroacetic acid containing sodium pyrophosphate to precipitate protein and incubated at 85°C for 20 min to hydrolyse nucleic acids. The precipitates were collected on glass-fibre filters, washed with 20 ml of 10% trichloroacetic acid–Na pyrophosphate and dried. Radioactivity was determined by liquid scintillation, and vertical bars show the range of duplicate incubations.

teins were further analysed by hydroxylapatite chromatography, which separates the proteins into three main fractions: Histones (unretained by the column and therefore eluting as a broad band) and two fractions of nonhistone proteins (eluted by 0.05 and 0.2 *M* Na phosphate, respectively) (MacGillivray *et al.*, 1972; our unpublished results following the same methods). The separation of ^{32}P-labeled chromatin proteins from 24- and 72-hr cultures are shown in Figs. 5A and B, respectively. Most of the incorporation is into the nonhistone fractions and there is a large increase in specific activity between 24 and 72 hr (Table 3). The specific activity of the histone fraction is low and actually decreases during culture. The 0.2 *M* nonhistone fraction has a higher specific activity than the 0.05 *M* fraction, which suggests that it contains more highly phosphorylated protein species. A similar but less dramatic difference has been observed in rat liver (Rickwood *et al.*, 1973). In 72-hr nuclei, a higher proportion of the total radioactivity appears in this more highly phosphorylated fraction (0.2 *M*; Table 3), which may reflect a qualitative change in the nuclear nonhistone protein complement. Even the high specific activity of the 0.2 *M* fraction shown in Table 3 is an underestimate since this fraction contains some RNA which contributes to the A_{280} (MacGillivray *et al.*, 1972). On the other hand, the low specific activity of the histones is actually overestimated relative to the nonhistones, because the histones lack tryptophan and so absorb less at 280 nm. For this reason, the ratio of nonhistones to histones on an A_{280} basis, as shown in Fig. 5, is also exaggerated relative to the true ratio in chromatin (Elgin and Bonner, 1970). The ineffectiveness of histone as a substrate has also been noted for isolated nuclear kinases (Takeda *et al.*, 1971; Dastugue *et al.*, 1974).

We conclude that increases in the degree of phosphorylation of chromatin-bound nonhistone proteins are largely responsible for the increases observed in whole nuclei. We now consider to what extent these increases are related to muscle cell differentiation in the cultures.

To test the possibility that the increase

TABLE 2

DISTRIBUTION OF PROTEIN KINASE ACTIVITY BETWEEN CHROMATIN AND SALINE-SOLUBLE FRACTIONS OF ISOLATED NUCLEI[a]

Conditions	^{32}P incorporated (cpm $\times 10^{-3}$)			Percent in chromatin [(cpm chromatin/cpm chromatin + saline-soluble) $\times$ 100]
	Whole nuclei	Chromatin fraction	Saline-soluble fraction	
Control	9.4 ± 1.4 (2)	8.7 ± 0.8 (3)	1.10 ± 0.07 (3)	89
+ Cyclic AMP (10^{-6} *M*)	11.9 ± 1.4 (2)	7.6 ± 0.7 (3)	1.44 ± 0.17 (3)	84
+ Casein (200 μg)	60.7 ± 6.4 (2)	46.7 ± 1.6 (3)	22.8 ± 0.6 (3)	67
+ Casein (200 μg) and cyclic AMP (10^{-6} *M*)	64.6 ± 3.2 (3)	36.6 ± 3.6 (3)	20.7 ± 1.8 (3)	64

[a] Nuclei from 70-hr cultures were suspended in chromatin buffer (0.14 *M* NaCl, 0.05 *M* Tris–HCl, pH 7.5) by gentle homogenization. Half of the suspension was then homogenized in a tight-fitting Dounce homogeniser and centrifuged at 15,000*g* for 15 min. The supernatant fluid was retained as the "saline-soluble" fraction, and the "chromatin" pellet was resuspended in an equal volume of chromatin buffer. Aliquots from each sample, consisting of or derived from 0.83 A_{260} units of nuclei, were assayed for phosphorylation activity as described previously (Nguyen thi Man *et al.*, 1974) except that the final ionic conditions in the assay were 0.06 *M* NaCl; 0.01 *M* $MgCl_2$; 0.08 *M* Tris–HCl, pH 8.0; 5 μCi of [γ-^{32}P]ATP. The average effect of cyclic AMP was + 3% (not significant), and the average recovery of enzyme activity after fractionation was 96%. Values are given ± standard errors of the means.

in phosphorylation activity is simply related to the increase in cell density during the *in vitro* culture period, cells were plated at three different densities and grown *in vitro* for 42 hr when they were harvested for the preparation of nuclei. Replicate plates were fixed for the determination of final cell density and the degree of cell fusion. As shown in Fig. 6, there is no significant increase in phosphorylation with increasing cell density over this range, which is comparable to the density range obtaining between 24 and 72 hr in time-course experiments (Fig. 1). Furthermore, Fig. 6 shows that the degree of cell fusion does increase with increasing cell density because cells fuse earlier at higher cell densities (Morris and Cole, 1972), so that phosphorylation changes are evidently not tightly coupled to either cell fusion or cell density.

Another possibility to consider is that the increase in phosphorylation activity may be shown by any cell type grown under these culture conditions. As a control cell type, we have used chick muscle fibroblasts obtained essentially free of myoblasts by successive trypsinization and replating as described in Methods.

Muscle-derived fibroblasts prepared by passaging in a similar way lose the ability to produce the creatine kinase isoenzyme characteristic of differentiated muscle (Turner *et al.*, 1974). As Fig. 7 shows, the results of the phosphorylation activity assays were in no way similar to those obtained with primary myogenic cultures.

In chick embryo thigh muscle, most cell fusion and muscle fibre formation occurs between the 11th and 18th day after fertilisation (Herrmann *et al.*, 1970). Figure 8 shows the change in nuclear protein phosphorylation in nuclei from whole thigh muscle between 11 and 16 days of embryonic development and, unlike the observations with cell cultures, phosphorylation decreases dramatically as development proceeds. In fact, this decrease closely parallels the decline in cytoplasmic protein kinase activity described by Piras *et al.* (1972) during chick embryonic thigh muscle development.

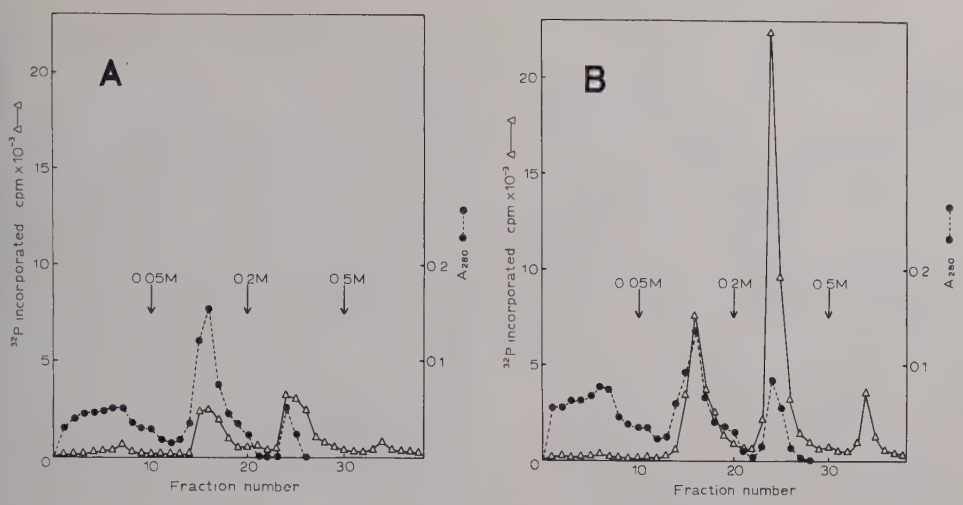

FIG. 5. Qualitative analysis of nuclear proteins by hydroxylapatite chromatography and changes during *in vitro* differentiation. Preparation of chromatin from nuclei isolated from 24-hr (A) and 72-hr (B) cultures and column chromatography are described in Materials and Methods. Fractions of 2.5 ml were collected and absorbance at 280 nm was determined (●——●). Each fraction was then made 10% in trichloroacetic acid (TCA) containing Na pyrophosphate and incubated at 85°C for 20 min to hydrolyze nucleic acids before collecting on glass-fibre filters and washing with 10% TCA for estimation of radioactivity (△——△). Arrows indicate the points at which the buffer was changed.

TABLE 3

SPECIFIC RADIOACTIVITIES OF PROTEIN FRACTIONS FROM HYDROXYLAPATITE COLUMNS AND CHANGES DURING CELL CULTURE

	Sodium phosphate concentration (*M*)	Specific activity (cpm/A_{280} unit)	Percent of total incorporation
24-Hr cultures	0.001	7,022	8.9
	0.05	17,527	33.8
	0.20[a]	157,987	43.9
	0.50	—	13.4
72-Hr cultures	0.001	4,123	3.3
	0.05	38,154	27.7
	0.20[a]	231,934	57.0
	0.50	—	12.0

[a] This fraction contains nucleic acid which contributes to the A_{280} so that the specific activity is an underestimate.

Since it was conceivable that the decrease in phosphorylation activity in embryonic thigh was occurring in different cell types from those isolated for cell culture, cell suspensions were prepared from embryonic thighs of different ages by the same mechanical disaggregation method used for standard cell cultures. Figure 8 also shows that a similar decrease in activity occurs in nuclei prepared from the isolated cells.

A possible argument that the decrease may be due to differential loss of enzyme or cofactors during preparation of nuclei is made less convincing by the observations of Piras *et al.* (1972) on cytoplasmic kinase and by our own observation that similar results are obtained if nuclei are prepared by an alternative method of homogenisation in 0.25 *M* sucrose followed by ultracentrifugation through a sucrose density gradient (results not shown).

Thus, it appears that nuclei from the same kinds of cell show an increase in phosphorylation activity during *in vitro* differentiation but a decrease during embryonic differentiation.

Measurements of ATP breakdown (Rickwood *et al.*, 1973) in 11- and 15-day embryonic nuclei under incubation conditions indicate that there is no increase in ATPase activity to account for the decrease in ^{32}P incorporation, and time courses of ^{32}P incorporation using embryonic nuclei fur-

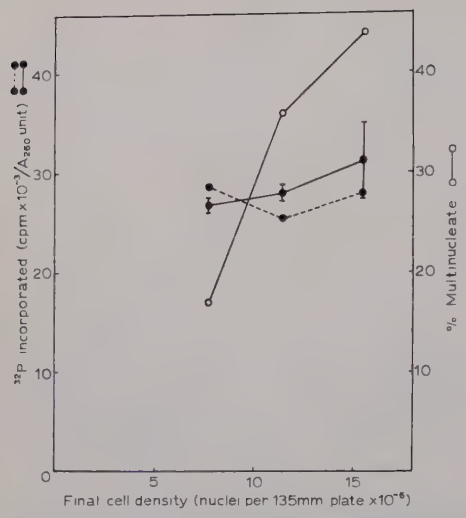

FIG. 6. The effect of final cell density on phosphorylation of nuclear proteins (●——●) and cell fusion (○——○). Cells were plated at different initial cell densities and harvested after 42-hr culture for isolation of nuclei. Replicate plates were fixed for determination of actual cell densities at 42-hr culture and the degree of cell fusion. The vertical bars show the standard errors of the triplicate incubations. Another separate experiment is also shown (●——●). Each incubation contained 0.50 A_{260} unit of nuclei.

ther suggest that the decrease is not due to increased phosphatase activity (results not shown).

DISCUSSION

Gene Activation during Myogenesis

Although it is generally accepted that muscle differentiation is a result of differential gene activity between myogenic cells and other cell types, it does not necessarily follow that changes in the pattern of gene transcription must occur during myogenesis *in vitro*, since these changes, or at least the preliminary changes in chromosome structure, may have already occurred before the myoblasts are removed from the embryo. However, work on creatine kinase activity (Shainberg *et al.*, 1971; Yaffe, 1971) and myosin mRNA synthesis (Buckingham *et al.*, 1974) has lead these authors to suggest that changes occur at both transcriptional and translational levels during *in vitro* myogenesis. Similar conclusions have been drawn from studies on myoglobin mRNA during embryonic muscle differentiation (Thompson *et al.*, 1973; Heywood and Kennedy, 1974) and from studies of developing chick oviduct (Comstock *et al.*, 1972). In the latter system evidence for changes in both species and conformation of nuclear nonhistone protein during development has been presented (Spelsberg *et al.*, 1973). Furthermore, the idea that "quantal" mitoses involve "derepressions of the genome" (Holtzer and Sanger, 1972) implies gene activation during the *in vitro* culture period.

Finally, direct studies of RNA synthesis during myogenesis by MAK (methylated albumin on kieselguhr) chromatography (Nguyen thi Man and Cole, 1972) and DNA–RNA hybridization (Nguyen thi Man and Cole, 1974) do reveal changes in the pattern of DNA transcription which are only partly due to changes in ribosomal RNA synthesis.

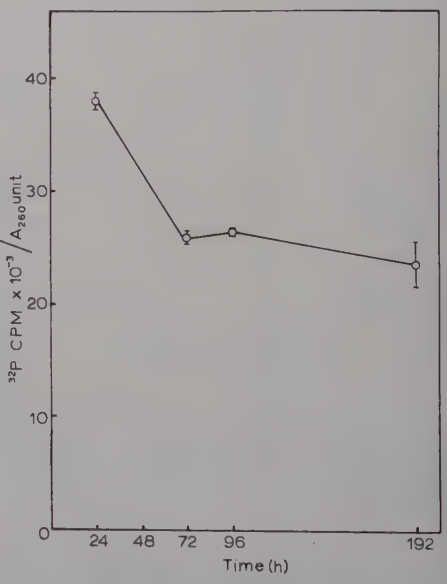

FIG. 7. Quantitative changes in nuclear protein phosphorylation during fibroblast culture. Preparation and culture of muscle-derived fibroblasts are described in Materials and Methods. Vertical bars represent the range of duplicate incubations and each incubation contained 0.67 A_{260} unit of nuclei.

Changes in Protein Phosphorylation Activity in Isolated Nuclei during Myogenesis

The incorporation of ^{32}P into nuclear proteins may be due partly to phosphate exchange as a result of a phosphorylation–dephosphorylation steady-state, partly to phosphorylation of proteins newly transferred to the nucleus at the time of isolation and partly to the production of new phosphorylation sites on the chromatin as a result either of the isolation procedure or of some ongoing conformational change at the time of isolation. The *rate* of protein phosphorylation measured in isolated nuclei need not necessarily reflect accurately the *degree* of phosphorylation of nuclear proteins in the intact cell at the time of isolation. Furthermore, one must bear in mind the probable loss, during isolation of nuclei, of low molecular weight factors which may modulate nuclear activity, such as cyclic AMP, and, perhaps, of regulatory proteins which normally go back and forth across the nuclear membrane (Gurdon and Woodland, 1970).

The increase in phosphorylation activity may occur in both myoblast and fibroblast nuclei, but it does not seem likely that fibroblasts alone make a major contribution to the increase. The low proportion of fibroblasts (16%) does not increase during the first 4 days of culture (after this time cultures become too dense to make a reliable estimate on the basis of morphology), so that the changes cannot be due simply to an increased proportion of highly active fibroblast nuclei.

The main reason for the increase in phosphorylation activity during myogenesis seems to be an increase in the activity of nuclear protein kinases, which is evident when the kinases are partially purified (Table 1). There is also a more tentative suggestion of an increase in the number of substrate sites (serine or threonine side chains per unit of chromatin) which could be due to an increase in the nonhistone protein component of chromatin or to a change in chromatin conformation, but this possibility will require more thorough investigation.

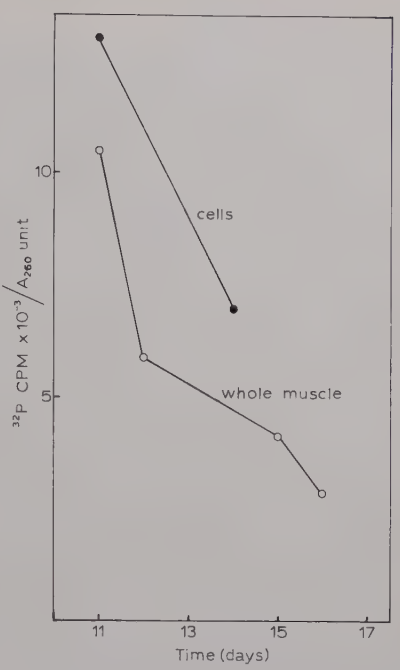

FIG. 8. Quantitative changes in nuclear protein phosphorylation activity during embryonic thigh muscle development *in ovo*. Nuclei were either isolated directly from thigh muscle at different days of development or obtained from myogenic cell preparations derived from embryonic muscle at different days of development as described in the Methods section. Each incubation contained 0.6 A_{260} unit of nuclei, and the maximum range of the duplicate incubations was ±10%.

On the evidence of Fig. 3, protein phosphatase activity seems unlikely to be sufficiently high during the standard 5-min incubation period to make a significant contribution to the level of ^{32}P incorporation. Although Fig. 3 also shows that ATPase activity is high in the nuclear preparations, there are no changes during myogenesis sufficient to change the rate of hydrolysis of [γ-^{32}P]ATP under our conditions, since the shape of the time course of ^{32}P incorporation is unaltered during myogenesis (Nguyen thi Man *et al.*, 1974).

The Role of Cyclic AMP

Although total phosphorylation activity in muscle cell nuclei is not affected by either cyclic AMP or cyclic GMP (Nguyen thi Man *et al.*, 1974), multiple protein kinases are known to exist in liver nuclei (Takeda *et al.*, 1971; Ruddon and Anderson, 1972), and it has been suggested that some of these are stimulated by cyclic AMP while others are inhibited (Kish and Kleinsmith, 1972) but there is no overall effect. However, as Fig. 2 shows, although minor protein kinases undoubtedly exist in embryonic muscle chromatin, they do not make a significant contribution to the activity that we assay. These minor components may, nevertheless, because of possible substrate specificity, make a major contribution to control of gene expression, and it is also possible that the main peak of Fig. 2 is not a single enzyme species. The kinase activities in the chromatin and saline-soluble fractions of muscle cell nuclei (Table 2) could perhaps represent different enzyme species, but, if so, then neither is stimulated by cyclic AMP.

The possibility that cyclic AMP independent kinases are in fact dependent kinases from which the regulatory subunit has become detached during preparation has been discussed by Walsh and Ashby (1973). These authors concluded that liver chromatin protein kinase was not of this kind since it was not inhibited by regulatory subunit, although, more recently, some inhibition has been obtained which could be partially relieved by cyclic AMP (Rikans and Ruddon, 1974).

Liver chromatin kinases also prefer casein to histone f2b (serine rich) as substrate, while cyclic AMP-dependent kinases show a marked preference for histone (Walsh and Ashby, 1973). Chromatin kinases from muscle cultures show a similar preference for casein (unpublished results), and the same preference by "cytoplasmic" kinase from early (11 day) embryonic thigh muscle can be seen in the results of Piras *et al.* (1972), whereas th lower activity of adult muscle shows som dependence on cyclic AMP and a greate preference for histone. However, it mu be emphasized that casein is not the no mal substrate for chromatin protein k nases, and that its presence has bee shown to disturb the normal interaction between cyclic AMP and some of thes enzymes (Kish and Kleinsmith, 1974).

Thus, although one cannot exclude role for cyclic AMP in the control of NH protein phosphorylation, the importance such a role has yet to be demonstrate convincingly.

The Pattern of Nuclear Protein Phospho rylation under Assay Conditions

Most of the ^{32}P incorporated into protei *in vitro* remains associated with the chro matin, only about 5% being removed b two saline washes (Fig. 4). The question to what extent saline-soluble or "nucle plasmic" proteins prepared in this way di fer from the chromosomal proteins re mains unclear and somewhat controver sial. Some workers (Comings and Tach 1973) maintain that most of the saline soluble proteins are identical with thos remaining in the chromatin fraction an that repeated washing will remove mor and more of these proteins, whereas Mac Gillivray and Rickwood (1974) have show that major differences can be revealed b two-dimensional gel electrophoresis.

Most of the ^{32}P incorporation associate with chromatin is in the nonhistone pro teins (Fig. 5 and Table 3). The specifi activity of histones decreases during myo genesis while that of the nonhistone frac tions increases. This also demonstrate that the increase in ^{32}P incorporation i not simply due to an increase in the nu clear concentration of nonhistone protein during myogenesis.

The shift in the pattern of nonhiston protein phosphorylation towards the mor highly phosphorylated fraction during my

genesis may suggest the appearance of ore serine- or threonine-rich proteins dur- g this time or increased accessibility of ese amino acids to the enzyme, but it is ssible that it may simply reflect the in- eased protein kinase activity.

Phosphorylation Related to Cell Fu- sion?

The increases in phosphorylation activ- y and cell fusion during culture *in vitro* o not demonstrate convincingly any close lationship between the two processes Nguyen thi Man *et al.*, 1974; and Fig. 1). he experiments of Fig. 6, in which the ercentage of fused cells increases three- ld from 16 to 43%, demonstrates that an ncrease in cell fusion is not a sufficient ndition for an increase in nuclear pro- in phosphorylation activity, and the situ- tion is not analogous to that suggested for lationship between cell fusion and crea- ne kinase activity (Shainberg *et al.*, 1971; urner *et al.*, 1974). Whether the increase phosphorylation activity can occur hen cell fusion is inhibited remains to be etermined.

Phosphorylation Related to Cell Density or Cell Division?

The density experiment (Fig. 6) and the broblast experiment (Fig. 7) both show hat the increase in nuclear phosphorylation activity is not due to increasing cell ensity *per se*. These experiments also suggest that the increase is not associated with cells entering a density-related stationary phase of growth, supporting evidence against this being that increases occur between 1 and 3 days when cells are still growing rapidly (Morris and Cole, 1972) and that cells were fed with fresh medium daily after the 2nd day of culture.

Riches *et al.* (1973) have shown that nuclear protein phosphorylation increases when Yoshida ascites cell cultures reach a stationary phase of growth and have suggested a relationship with the observation that a greater percentage of the nuclear RNA in stationary cells is able to hybridize to repeated DNA sequences than in growing cells. In muscle cells, however, it is the percentage of pulse-labeled RNA hybridising to *rare and unique* DNA sequences that increases during myogenesis, as phosphorylation increases (Nguyen thi Man and Cole, 1974), so it would seem that the increases in phosphorylation activity in these two systems are rather different phenomena.

As cell fusion proceeds in culture, the proportion of dividing cells must decrease, an impression confirmed by studies of thymidine incorporation into DNA (O'Neill and Stockdale, 1972), but, on the evidence of Figs. 6 and 7, this process is not related to the increase in nuclear protein kinase. As expected, thymidine incorporation does fall as cell fusion increases in density experiments of the type shown in Fig. 6 (unpublished).

In intact 12-day embryonic thigh muscle, most of the single cells appear to have stopped dividing (Herrmann *et al.*, 1970) but, when cell preparations from this tissue are placed in culture, rapid proliferation soon begins. Could the increase in nuclear protein phosphorylation be induced by this initial stimulation of division in the cell population? The *biosynthesis* of nonhistone proteins is known to increase when density-inhibited fibroblasts are stimulated to divide by a change of medium (Rovera and Baserga, 1971). However, this does not appear to be true of *phosphorylation* of nonhistone proteins when confluent chick fibroblasts are replated at lower densities in fresh medium (Fig. 7).

Is Phosphorylation Related to Gene Activation?

The results discussed so far are not inconsistent with a hypothesis that the increase in phosphorylation activity is specifically related to the course of cell differen-

tiation in muscle cultures. Our observations on nuclear phosphorylation in the developing embryo (Fig. 8), however, are more difficult to reconcile with this hypothesis. Indeed, it would seem that neither the overall increase in phosphorylation activity observed *in vitro* nor the overall decrease *in ovo* is a necessary condition for, or consequence of, the development of functional muscle from its single cell precursors.

Piras *et al.* (1972) observed a decline in kinase activity during embryonic development, similar to that of Fig. 8, in a muscle extract prepared by homogenization followed by a centrifugation step which would remove intact nuclei. It is conceivable that we are dealing here with the same enzyme, since in rat liver the principal nuclear kinase is also found in the cytoplasm (Dastugue *et al.*, 1974).

Our experiments and these of Piras *et al.* (1972) are consistent with a rapid decay of protein kinase activity beginning at about 11 days of incubation with a half-life of about 3 days. An alternative explanation could be the accumulation of an inhibitor of enzyme activity, but Piras *et al.* (1972) have argued against this possibility for the enzyme activity they studied.

As in the case of muscle cultures, we do not favor the idea of a close connection between the changes in phosphorylation activity and changes in cell division and cell fusion, though the arguments against such relationships are less firm. Cell division in the embryonic thigh estimated by an autoradiographic method seems to decline earlier (Herrmann *et al.*, 1970) than protein kinase activity. Attempts to relate cell division to measurements of DNA polymerase activity in cell-free systems are complicated by the presence of catabolic enzymes (Stockdale, 1970), but some relationship between the declines in protein kinase and DNA polymerase activities may exist (Piras *et al.*, 1972). Our cell preparation procedure removes the larger myotubes and yet a similar decline in enzym activity occurs in such preparations (Fi 8), but it is not yet clear whether the d cline occurs in unfused cells as well as small myotubes.

It may be that inhibitory factors accum late in the intact embryo to bring abo the decline in kinase activity and tha when the muscle cells are removed to t *in vitro* environment, the enzyme can b gin to accumulate. Hypotheses of this kin are testable and experiments are in pro ress. These considerations, perhaps, mak less clear the possible role of changes nuclear protein phosphorylation in ge activation during cell differentiation, b they cannot, of course, rule out such a rol Rather, they point towards experimen on possible multiple forms of the enzym with different specificities or towards stu ies of changes in the phosphorylation specific nonhistone proteins, as opposed the work on overall changes described this paper.

This work was supported by grants from the Mu cular Dystrophy Group of Great Britain and t Medical Research Council. We thank Dr. A. MacGillivray and Dr. D. Rickwood for their invalu ble advice, Miss Margaret Berry and Mrs. Melan Piper for skilled technical assistance and Mrs. Sal White for preparing the illustrations.

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The Amoebal Cell of *Physarum polycephalum:* Colony Formation and Growth

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Two stages of colony growth were observed during microscopic studies of *Physarum polycephalum* amoebae. During the first stage, "spreading growth," the colony is composed of dispersed single cells. During the second stage, "aggregate growth," most of the active cells in a colony are aggregated in a ring at the colony boundary. Measurements of cell movement as a function of bacterial concentration indicate that, during both spreading and aggregate growth, cell movements are not affected by changes in bacterial concentration but that the transition from spreading to aggregate growth occurs earlier on plates with lower bacterial concentrations. These results indicate that autonomous characteristics of the amoebae are more important for the determination of colony form than local variations in the concentrations of nutrients.

The genetic determination of colony form is demonstrated by the existence of mutants that display specific alterations in colony morphology. Because the aggregate rings of these mutants move at an increased rate, mutant clones appear as variant sectors of wild-type colonies. The increased rate of mutant ring movement suggests that this selection method may be a useful technique for isolating mutant myxamoebae with defects in movement and behavior.

INTRODUCTION

The myxomycete *Physarum polycephalum* is an organism that offers opportunities for studies of the molecular mechanisms involved in eukaryotic cell movement. During the different stages of its life cycle, *P. polycephalum* exhibits a variety of motile phenomena including rapid protoplasmic streaming, amoeboid movement, flagellar propelled swimming, and the protoplasmic movements involved in the formation of stalked sporangia (Gray and Alexopoulos, 1968). Moreover, plasmodia are known to contain large quantities of actin and myosin, and techniques are available to purify these proteins in milligram quantities from plasmodial homogenates (Adelman and Taylor (1969). Finally, the results of recent studies of the genetic system of *P. polycephalum* suggest that the techniques of molecular genetics can be combined with phenomenological and biochemical techniques to study the molecular basis of the motile phenomena of *P. polycephalum* (Dee, 1966; Haugli and Dove, 1972).

Processes that involve movement in response to a stimulus are often particularly suitable for genetic studies because of the possibility of direct selection of mutants. These selections are generally achieved by isolating deviant individuals that become spatially separated from the wild type because they fail to respond properly to a stimulus. Selection procedures of this kind have been used successfully to isolate mutants of *Escherichia coli* (Armstrong *et al.*, 1967), *Drosophila melanogaster* (Benzer, 1967) and *Paramecium aurelia* (Kung, 1971). This communication presents the results of some initial studies directed toward the development of procedures of this kind for the selection of mutant *P. polycephalum* amoebae with defects in cell movement and behavior.

MATERIALS AND METHODS

Culture Conditions

(*a*) *Myxamoebae.* Amoebae were cultured on lawns of live or formalin-killed *E.*

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coli on agar or on Millipore filters (Haugli and Dove, 1972). Three different media were used for the agar plates: 0.05% Oxoid liver infusion (LIA); the semidefined medium of Daniel and Baldwin (1964), diluted tenfold (SD/10); and McIlvaine's buffer at pH 5, diluted tenfold (pH 5) (Collins and Tang, 1973).

(*b*) *Matings*. Matings were carried out by inoculating with toothpicks amoebae of different mating type onto a pH 5 agar plate spread with 10^{10} formalin-killed bacteria.

(*c*) *Plasmodia*. Microplasmodia were transferred from the mating plate to an SD/10 agar plate spread with 10^{10} formalin-killed bacteria. After plasmodia had begun to grow vigorously they were transferred onto a piece of filter paper (Schleicher and Schuell 576) suspended on a stainless-steel grid over diluted semidefined medium (SD/2).

(*d*) *Sporulation*. Plasmodia were allowed to starve on SD/2. One to two days after the initiation of starvation a series of illuminations was begun. The plasmodia were illuminated 4 hr each day until sporulation occurred.

(*e*) *Germination*. Sporangia were broken up as described by Haugli *et al*. (1972), except that phosphate-buffered saline (PBS) was used instead of water (Goodman, 1972). Spores were left suspended in the PBS for 6–24 hr before swimming amoebae were observed.

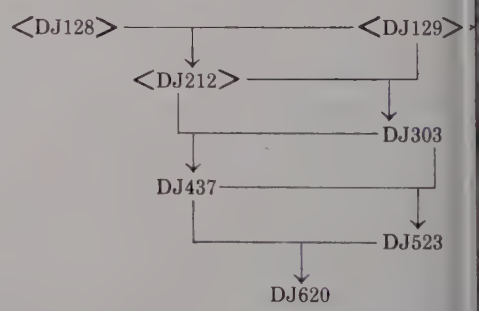
Strains

The strains used in this work were constructed by an inbreeding procedure from the Wis 1 plasmodial isolate of Dr. M. Backus. The traits which were maintained in this procedure were those stimulating mating, sporulation, and germination. The two amoebal strains which differ in mating type but are partially inbred for these traits are DJ437 (*mt* 2) and DJ620 (*mt* 1, cyc^R).

The protocol used for this inbreeding was constructed on the principle of fir selecting for segregants of Wis 1 whic mated, sporulated, and germinated eff ciently and then carrying two such segr gants through a series of offspring younger parent inbreedings.

The first step was accomplished by pla ing amoebae issuing from Wis 1 on SD/ plates at concentrations from 10^2 to 10^4 po plate. Forty microplasmodia were picke as soon as they were visible, and each w tested for ability to sporulate and germ nate. The best plasmodium was then use to initiate a second cycle of selection ju like the first. From this, two founding pa ents were picked, DJ128 and DJ129. Thes parents mated well and gave plasmodi that sporulated and germinated well.

Inbreeding was then performed by ty ing for mating specificity the amoebae d riving from a plasmodium and then ma ing an amoebae with its younger paren In this way, the inbred partners DJ437 ar DJ620 were produced:



Measurements of Colony Size

Colonies lacking boundary rings we measured from photomicrographs. The micrographs were taken through the 2. power bright field objective of a Zeiss GF microscope. The two diagonals of the micr graph were drawn, and the diameter of th colony was taken parallel to each diag nal. These two values were then average to give the colony diameter. When only portion of the colony was captured in th micrograph, the radius was determined b estimating the locus of the center of th

olony from a ring of aggregated cells inde such a colony.

Colonies with boundary rings were measred directly on the agar plate, by juxtaosing a plastic ruler to the back of the late. Except where noted, these measurements were made on four or five colonies nd the results averaged.

Mutagenesis

We have used the method of Haugli and Dove (1972) for mutagenesis by ethyl methne sulfonate (EMS) on cells growing on Millipore filters. EMS was dissolved in .05 *M* phosphate, pH 7.

RESULTS

Stages of Colony Growth

When amoebae are cultured on agar surfaces, they show two distinct modes of colny growth. In the first stage, which we all "spreading growth," colonies consist of dispersed single cells (Fig. 1). During this tage no encysted cells are present. Therefore, all of the cells in a colony of this stage appear to be active.

In the second stage, which we call "aggregate growth," colonies are bounded by a ring of aggregated cells (Fig. 2). This structure projects upward from the agar surface and appears to maintain a constant size as the colony expands. During this stage most cells observed inside the aggregate ring are dormant cysts. Thus during aggregate growth most of the active cells are part of the ring.

Spreading Growth and the Transition to Aggregate Growth

The movements of cells away from the center of a colony during spreading growth could be either random or directed. Two kinds of mechanisms can be imagined for directed movements. On the one hand, the cells could be moving toward a chemoattractant. In this case the spreading movements would be along gradients created as the amoebae consumed bacteria. On the other hand, the movements could be the results of chemical interactions among cells which caused them to repel each other.

FIG. 1. Eighty-nine hour colony on H_2O agar plates (pH 5). Scale bar, 200 μm.

One way to distinguish between chemoattractant mechanisms and others is to observe the behavior of amoebae during colony formation in the presence of different concentrations of bacteria. Known mechanisms for sensing chemoattractants or light are highly nonlinear, resulting in responses that are proportional to the ratio of the concentration change to the background level (Stevens, 1970; Feinleib and Curry, 1971; Dahlquist *et al.*, 1972). Thus, if the spreading movements were a response to a gradient in bacterial concentration, more movement would be expected on plates with fewer bacteria.

The data in Fig. 3 show that the radial movement of amoebae during spreading

growth is not affected by a 16-fold variation in the bacterial concentration. Moreover, at a bacterial concentration a factor of two lower (L/32 plates) where amoebae create observable gradients of bacterial concentration within 3 days, the rate of outward movement of the amoebae is actually slower. Thus the radial movement of the amoebae during spreading growth does not appear to be caused by chemoattraction.

However, nutrient exhaustion is correlated with a different change in amoeba behavior, the transition to aggregate growth. This transition is observed most clearly on L/16 and L/32 plates. As the bacteria in the center of a colony are exhausted, amoebae began to move radially and to concentrate on the edge of the bacterial lawn. This process results in the formation of a ring of cells aggregated inside the colony at the border of the bacterial lawn (Fig. 4). As is indicated in Fig. 3, the ring forms later on plates with higher bacterial concentrations, although the density of amoebae in the center of colonies on these plates continues to increase. The transition to aggregate growth is completed by expansion of the ring to form the colony boundary.

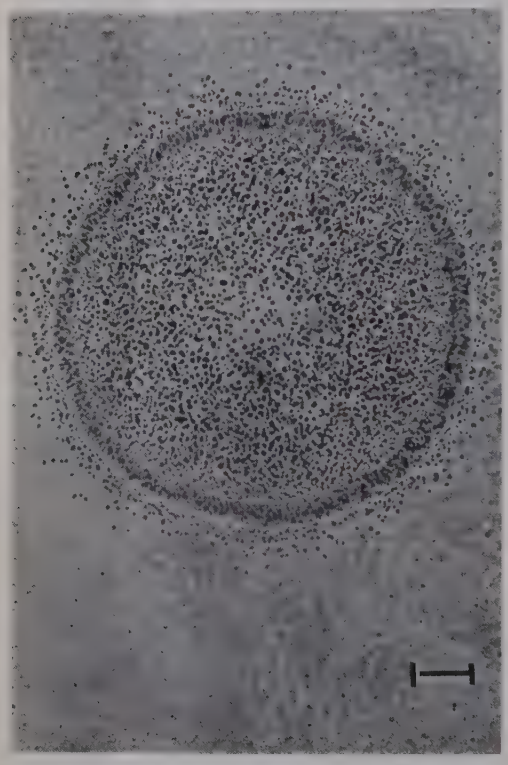


FIG. 2. Six-day (144-hr)-colony on LIA plates. Scale bar, 200 μm.

Aggregate Growth

Microscopic studies of aggregate growth yield two observations about the behavior of ring amoebae. One is that the ring and the edge of the bacterial lawn always coincide. Thus the advance of the ring must be coordinated with nutrient depletion. The other observation is that the width of the ring does not change measurably as the radius of the colony increases. Since the cells in the ring are tightly aggregated, we

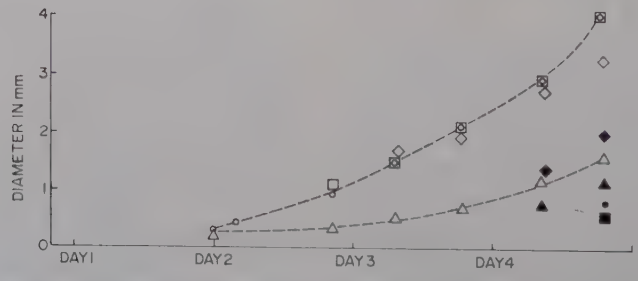


FIG. 3. Colonies of amoebae growing on agar plates (pH 5) inoculated with varying quantities of live *E. coli* were photographed at the times indicated. The diameters of colonies and of aggregate rings, if present, were measured as described in Materials and Methods. Open symbols represent colony diameters and closed symbols represent ring diameters. The *E. coli* concentrations were dilutions of L = 2×10^{10}/ml. —□—, L; —○—, L/4; —◇—, L/16; —△—, L/32.

FIG. 4. One-hundred-four-hour colony on H_2O agar plates (pH 5) inoculated with L/16 bacteria. Scale bar, 200 μm.

infer that the number of cells in the ring rises in simple proportion to the radius of the colony. The additional cells generated by amoebal growth are left behind in the interior of the colony as inactive cysts.

In order to characterize the process of ring expansion in more detail, colony diameters were measured as a function of time and bacterial concentration. These measurements (Table 1) show that after a colony reaches a macroscopic size (ca. 2–3 mm in diameter), its ring expands at a constant rate of 20–40 μm/hr. This expansion must involve cell migration in addition to cell division, since the latter process would require 8 hr to create an additional cell (10–20 μm in diameter).

The data in Table 1 show that the rate of these movements is not appreciably affected by large variations in the concentration of bacteria present on a plate. In one experiment the rate of ring expansion varied less than twofold over a 64-fold variation in the concentration of live bacteria. In a second experiment there was no significant change in the rate of ring expansion over a 125-fold variation in the concentration of formalin-killed bacteria. Again, the failure to observe increased cell movements at lower nutrient levels suggests that the movements are not controlled entirely by a mechanism for movement up chemoattractant gradients.

TABLE 1

RATE OF RING EXPANSION IN MYXAMOEBAL COLONIES AS A FUNCTION OF BACTERIAL CONCENTRATION

Concentration of live bacteria[a]	Rate of ring diameter increase (mm/day)	Concentration of dead bacteria[b]	Rate of ring diameter increase (mm/day)
L[c]	2	D[c]	0.8
L/4	1.4	D/5	0.9
L/16	1.2	D/25	0.9
L/64	1.3	D/125	0.9

[a] Strain DJ129 was used for the experiment with live bacteria on pH 5 plates.

[b] Strains DJ437 was used for the experiment with dead bacteria on pH 5 plates.

[c] L, 2×10^{10} live *E. coli*; D, 3×10^{11} formalin-killed *E. coli*.

Genetic Variation in Colony Morphology

In their initial studies of mutagenesis of *P. polycephalum* amoebae, Haugli and Dove (1972) used plaque size as a genetic marker. The results of the studies presented above suggest a selection method to obtain a second class of colony morphology mutants.

It is possible that mutant amoebae that move more rapidly during ring expansion would have an enhanced capacity to move to new sources of nutrient. This kind of mutant would be more likely than the wild type to stay active during aggregate growth and, therefore, would have a selective advantage over wild type in mixed colonies. Thus, a clone of mutants growing in a mixed colony would be expected to form a sector of the colony revealed by a

segment of the aggregate ring expanding at an unusually rapid rate.

In fact, sectors that display such colony alterations can often be observed in colonies of amoebae that are allowed to grow to large size. Such sectors generally display a more rapid rate of ring expansion and a different pattern of encysted cells than the rest of the colony. In addition the aggregate ring of the sector may display a variant morphology.

The idea that these sectors are formed by clones of mutant cells is supported by the finding that the frequency of the appearance of sectors is significantly increased by mutagen treatment. Five sectors appeared in 20 mutagenized colonies, each formed from 2.5×10^5 cells that had been treated with 2% EMS, as described in Materials and Methods. In comparison only six sectors appeared in 176 parallel colonies of nonmutagenized cells.

Four strains, DJ-1, DJ-2, DJ-3, and DJ-8, obtained by cloning variant sectors of DJ437, were used for more detailed studies. Clones of all four of these strains form colonies that are readily distinguishable from wild type. As expected, one common characteristic of the variant is an increased rate of ring expansion (see Fig. 5 for data on DJ-3 and DJ-8).

Moreover, reconstruction experiments show that all four of these strains have a selective advantage over wild type during aggregate growth. The results of these experiments are presented in Table 2. In each case colonies were formed that included a small number of variants among a large population of normal cells. In all cases sectors derived from the variant were recovered. Since the doubling time is longer for the variants than for wild type, the selective advantage of the variants is probably the result of increased access to nutrients rather than more rapid use of nutrients for cell growth.

The results of the reconstruction experiments suggest that the frequency of detection of 10^{-6} for sector variants after EMS

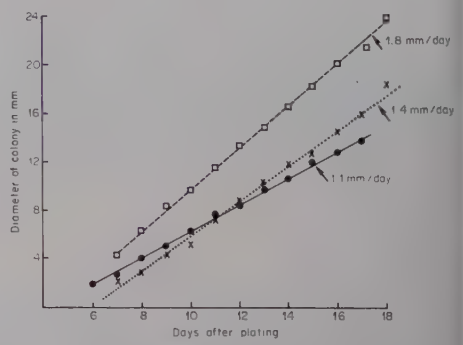


FIG. 5. Ring expansion during aggregate growth was measured for colonies growing on LIA plates on live bacteria as described in the Materials and Methods. —●—, DJ437; —□—, DJ3; —×—, DJ8.

TABLE 2

RECONSTRUCTION OF COLONY MUTANT SELECTION IN MIXED POPULATIONS OF MUTANT AND WILD-TYPE CELLS[a]

Strain	Doubling time (hr)	Number of cells per colony		Number of colonies tested	Number of sectors recovered	Equivalent selection per dish
		W.T.	Mutant			
W.T. (DJ437)[b]	8.7	2.5×10^5	—	176	6	—
DJ1	13.2	6×10^4	200	8	50	7.5×10^3
DJ2	11.4	1.2×10^4	100	12	22	8.8×10^2
DJ3	11.2	1.9×10^5	12	40	25	3.8×10^2
DJ8	14.4	2.2×10^5	14	40	8	1.1×10^4

[a] Four colonies were tested per agar dish. The colonies were formed by spotting a mixture of mutant and wild-type cells in a 5 μl-drop. The "equivalent selection" is the ratio of wild-type cells to mutant cells necessary to recover one mutant sector per dish.

[b] W.T., wild type.

mutagenesis is equivalent to a frequency of occurrence on the order of 10^{-4}. In the same mutagenesis experiment one cycloheximide mutant was found among 5×10^6 cells tested. Thus, sector variants seem to be several orders of magnitude more common than cycloheximide-resistant mutants.

Analysis of the segregation of the parental phenotypes among progeny of a cross of DJ8 and wild type has confirmed that the variant phenotype of DJ8 is caused by a hereditary mutation. The most striking aspect of the phenotype of DJ8 is the failure of DJ8 colonies to form a continuous ring that rises above the agar surface (Fig. 6). When progeny of DJ8 × DJ620 were observed a number of clones with this phenotype were identified. However, the majority of progeny formed colonies that were different from either parent. These colonies eventually formed rings, but at a later time than wild type. Since both parental phenotypes appeared about one-fourth of the time (Table 3), the DJ8 phenotype is probably caused by changes in two unlinked genes.

FIG. 6. Eight-day (192-hr)-colony of DJ8 on LIA agar plates. Scale bar, 200 μm. Single cells are present at the colony edge. The interior of the colony is composed of single cells and small aggregates.

Two of the other variants were not amenable to genetic analysis because plasmodia formed by crossing them with wild type were deficient in sporulation or germination. In the third case, DJ3, it was possible to recover progeny from a cross with wild type. However, only 18 of 77 progeny clones displayed a variant phenotype. Moreover, none of these phenotypes was similar to DJ3. In contrast to the rapid rate of expansion of DJ3 colonies (Fig. 5) all of the progeny colonies expanded at

TABLE 3

SEGREGATION OF PROGENY OF A CROSS BETWEEN A COLONY MUTANT AND WILD TYPE[a]

Clones	Number of colonies	Number of wild type	Number ambiguous	Number DJ8-like	Number ambiguous
Progeny of DJ8 × DJ620					
Clones on LIA plates	42	7	2		
Toothpick colonies on SD/10 plates	108			26	12
Control progeny of DJ437 × DJ620					
Toothpick colonies on LIA plates	23	20	3		
Toothpick colonies on SD/10 plates	45			0	0

[a] Progeny clones were tested for one parental phenotype either by plating small numbers of amoebae so that discrete colonies were obtained or by inoculating a number of the amoebae at a single spot with a toothpick. Colonies similar to wild type were scored as ambiguous if a halo of cells was present outside an otherwise normal aggregate ring. DJ8-like colonies were scored as ambiguous if there was some indication of a disorganized ring.

rates that were slower than wild type. Thus, the nature of the lesion present in these three variants is still unclear.

DISCUSSION

Two central results emerge from the studies reported above. First, large changes in nutrient conditions produce little change in the behavior of *P. polycephalum* amoebae. Second, it is possible to isolate mutant strains which form amoebal colonies that can be reliably distinguished from wild type. Both of these results support the conclusion that the cell movements which determine the pattern of cells in *P. polycephalum* colonies reflect the genotype of the strain involved more than small variations in the nutrient conditions. This conclusion does not imply that amoebae are unable to respond to changes in bacterial concentration. However, the responses of amoebae to variations in bacterial concentration only appear to perturb the standard response of the amoebae to the presence of bacteria: To systematically consume the available bacteria.

The results reported above indicate that it is possible to analyze the process of colony formation with genetic techniques. However, only one of the four variants studied was suitable for genetic experiments. This result appears to be an artifact of the tendency to choose strains for detailed studies that display the most obvious colony abnormalities. The three strains that were not suitable for genetic analysis all formed colonies with more obvious abnormalities than DJ8. Moreover, the observation that the cells of all three of these strains have cross sections that are two to three times longer than wild type suggests the possibility that these strains had each undergone a ploidy change. Finally, the results of Adler and Holt (1974) with *P. polycephalum* and those of Fulton (1970) with *Naeglaria* indicate that changes in ploidy can cause changes in colony form similar to those observed. Therefore, it should be possible to increase the proportion of useful mutants by choosing those with normal cell size.

The isolation and characterization of additional mutants of this kind should help in the analysis of the cellular responses involved in colony formation. In addition, these mutants may be useful for studies of the plasmodial stage of the *P. polycephalum* life cycle. One conjecture is that the acellular macroplasmodia evolved from colonies of interacting amoebae. If this hypothesis were correct, characterization of the pleiotropic effects of amoebal mutants upon the plasmodial stage of the life cycle would also improve our understanding of the mechanisms which determine the morphology and behavior of plasmodia.

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Control of Insulin Secretion in the Developing Pancreatic Rudiment[1]

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The embryonic rat pancreas, removed on the 14th day of gestation and cultivated *in vitro*, accumulates differentiated levels of exocrine enzymes and insulin. In the period corresponding to days 16–22 *in vivo*, 99% of the final insulin content accumulates. During this period we have studied the development of competence for insulin secretion, the regulation of this secretion by glucose and other secretatogues, and the rate of synthesis following a secretory challenge. Our results demonstrate that the capacity for insulin secretion develops in parallel with the accumulation of insulin in secretory granules since β granules appear at day 16. On day 16, after 48 hr of culture, both glucose and caffeine are required for detectable insulin secretion. At later stages, insulin release can be effectuated by glucose alone. In the fetal pancreas at day 20 of development, glucose is ten times more efficient than caffeine and fourfold more efficient than caffeine combined with either glucagon, cholera toxin or dibutyryl cyclic AMP. Glucagon, cholera toxin or cyclic AMP in the presence of caffeine increases equally (about tenfold) both the "basal" and the glucose-induced level of secretion. This suggests that glucose and caffeine act independently but synergistically. The integrity of the cells is maintained under the stimulation conditions, and there is a selective increase in insulin synthesis measured during 18 hr following stimulation of insulin release.

INTRODUCTION

The differentiation of exocrine and endocrine cells involves morphological and biochemical changes, including the accumulation of specific products and the competence to secrete these products in response to specific stimuli. The development of the competence to secrete insulin, then, is of particular significance, because this hormone may play a role in development.

The morphogenesis and the accumulation of the specific products of the rat pancreas *in vitro* are qualitatively similar to that occurring *in vivo* (41, 45, 46, 54, 56). For this reason, *in vitro* studies can be effectively used to follow developmental events. Insulin, like the exocrine enzymes, accumulates in a biphasic fashion (11, 45, 47, 56) and is first found when the pancreatic bud appears (20–22 somites; 11 days of gestation in the rat); the specific activity of insulin per cell remains low and relatively constant for several days (protodifferentiated state). Between days 14 and 15, there is a dramatic increase in the rate of insulin accumulation. In a few days, this leads to differentiated levels of insulin, approximately 1000-fold higher than that of the protodifferentiated state. This second rise in insulin content is due to an increased rate of synthesis (11, 48) and coincides with the appearance of β secretory granules (45–47) in the insulin-producing B cells.

Glucose exerts a dramatic stimulation on insulin release in adults but is relatively ineffective during the perinatal period (1, 2, 18, 26, 59, 62, 63, 68). In *in vitro* experiments at this developmental stage, the release of significant amounts of insulin elicited by glucose and other natural secretagogues requires the presence of high levels of caffeine or its analogs. These

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xanthine derivatives are known to inhibit phosphodiesterase, the enzyme that hydrolyzes cyclic nucleotides; thus, the action of these compounds is thought to be due to enhancing the intracellular levels of the cyclic nucleotides (6, 29). High levels of insulin are found in the circulation of rat fetuses during the perinatal period (19) and the increase in insulin correlates with glucose elevation in maternal blood (3, 25). Thus the perinatal B cells appear to respond to glucose *in vivo*.

We have investigated the development of the secretory response of embryonic and fetal B cells to various secretagogues. The capacity for insulin secretion *in vitro* develops in parallel with the accumulation of this hormone in secretory granules. Under our conditions, glucose stimulates insulin release, although the release represents a smaller proportion of the total content than that found in the adult pancreas. Finally, following a secretory challenge, there is a long-lasting increase in insulin synthetic rate.

MATERIALS AND METHODS

Animals. Male and female Sprague–Dawley rats, fed *ad libitum* were kept paired in single cages. For the determination of gestational age, fertilization (day 0 of embryonic life) was assumed to occur at noon on the day the vaginal plug was discovered. With this method, the normal variance in developmental age between litters is 6 hr.

Reagents. Earle's balanced salt solution (EBSS),[4] Eagle's basal medium (BME) and antibiotic–antimycotic mixture (100×) were purchased from Grand Island Biological Co. ^{125}I-labeled insulin was obtained commercially from Amersham/Searle (1M-38). Insulin binding reagent (Lot K 4796) was purchased from Wellcome Reagent Ltd. Rat insulin (Lot R170, 21.4 U/mg) was a gift from Dr. Schlicktkrull from Novo Copenhagen. Crystalline porcine glucagon (Lot GLF 599A) was kindly donated by Dr. Mary Root (E. Lilly and Co., Indianapolis). Using a rat standard, in our immunoassay this glucagon showed an IRI contamination of less than 15 mol per μmol of glucagon. Cholera toxin, received from Dr. Northrup, Coordinator of the SEATO Cholera Research Program, NIAID, was prepared under contract from the National Institute of Allergy and Infectious Disease (NIAID) by R. A. Finkelstein, Ph.D. (the University of Texas Southwestern Medical School, Dallas, Tex.). Trasylol (Lot K2171 and RAE 6039-329) was a gift of Verfahrenentwicklung Biochemie, Bayer AG. [4,5-^{3}H]leucine was purchased from New England Nuclear. Calf thymus DNA, caffeine, α-D-glucose, crystallized bovine albumin (A grade) and dibutyryl cyclic AMP (A grade, Lot 010173) were purchased from Calbiochem.

Culture. Pancreatic rudiments were excised from 14-day-old embryos under sterile conditions and dissected in EBSS supplemented with 0.1% albumin. The rudiments were cultured on Millipore filter (0.25 μm, THWP 102 FO) platforms fixed on Polystyrene culture wells (Dispostrays MVC-96, Linbro of the Pacific, Los Angeles) which contained approximately 250 μl of BME. Unless otherwise indicated, the medium was supplemented with amino acids (7× BME), 2 m*M* glutamine, 10% chick embryo extract, 100 U/ml of penicillin, 100 μg/ml of streptomycin and 25 μg/ml of Mycostatin. The cultures were incubated for 2–8 days in 5% CO_2–95% air at 37°C. The medium was changed every 24 hours. Rudiments were harvested at appropriate times during the culture period, washed with EBSS and stored at −70°C in polyethylene Microfuge tubes (Beckman–Spinco) until assayed for protein, DNA and insulin content.

Insulin release into the culture medium. For studies on the levels of insulin released by pancreatic rudiments in culture,

[4] The following abbreviations are used: cAMP, cyclic adenosine monophosphate; Bt_2cAMP, dibutyryl cyclic adenosine monophosphate; BME, Eagle's basal medium; EBSS, Earle's balanced salt solution; IRI, immunoreactive insulin; TCA, trichloroacetic acid.

the medium was replaced with fresh medium supplemented with 0.1% albumin (instead of embryo extract) and 10,000 units/ml of pancreatic trypsin inhibitor (Trasylol). Substitution of embryo extract with albumin and Trasylol was necessary to avoid destruction of the hormone by proteolytic enzymes present in the embryo extract. After a 6-hr incubation period at 37°C in this modified culture medium, the medium was collected and stored at −20°C until assayed for insulin content. Under these conditions, added ^{125}I-labeled insulin was quantitatively precipitated by trichloroacetic acid, and the recovery of added cold insulin was greater than 90% as measured by immunoassay. Six-hour incubations in the albumin-containing medium had no measurable effect on the DNA, protein or insulin content of the rudiments.[5]

Stimulation of insulin secretion. Pancreases on their supporting filter were washed for approximately 2 min in EBSS supplemented with 0.4% albumin and transferred at 37°C into a culture tube (17 × 100 mm (2001) Falcon Plastics tubes) containing 1 ml of EBSS–0.4% albumin without (for the control) or with the desired secretagogue(s). Retaining the pancreases on their filter support avoids mechanical damage of the tissue and consequently increases the reliability of the experiments. Incubations were carried out at 37°C under a 5.6% CO_2–94.4% O_2 atmosphere (pH 7.4) with continuous agitation (150 rpm) in a rotary shaker (New Brunswick Scientific Co.) for 2 hr unless otherwise stated. At the end of the incubation period the medium was stored at −20°C until assay; the pancreases were either stored at −70°C for later assays or fixed for ultrastructural analysis. Labeled insulin was quantitatively recovered from the incubation medium in the presence or absence of a proteolytic inhibitor (Trasylol, 2400 kIU/ml) as calculated by a paired t test ($t = 0.17$, $n = 10$). Therefore, Trasylol was ordinarily omitted from the incubation medium. The Millipore filter does not bind labeled insulin or interfere with the determination of the insulin released.

Assays. DNA content was measured using a micromodification of Burton's technique (7). Proteins were measured according to a modification (55) of the method of Lowry *et al.* (32). Insulin was assayed by a double antibody radioimmunoassay technique (38) with rat insulin as a standard. Assays were performed directly on culture media. Pancreatic rudiments were sonicated in acid ethanol and extracted overnight, after which the supernatant fluids, were lyophilized. The extract was reconstituted in the immunoassay buffer. In the assay used, 15% of the bound radioactive insulin was displaced by 0.8 ng/ml of cold rat insulin. The standard curve was linear between 0.8 and 20 ng/ml when the logit of the percentage of the radioactivity bound in the presence of cold insulin is plotted against the logarithm of the amount of cold insulin added. The comparison of the unknown sample values with the standard curve was carried out with a PD P-12 computer (Digital Equipment Co.). All values were measured using two or three dilutions falling on the linear portion of the standard curve, and the values were then proportional to the dilution. A single batch of antibodies was used for all determinations. All insulin assays were controlled by the addition of an internal standard.

Analysis of insulin secretion. Each point represents the average of at least six determinations, unless otherwise stated. The experimental error in the analysis of the variance for insulin secretion ranges between 3 and 15%. The experimental error for protein and DNA assays are 1.7% and 2.6%, respectively. The statistical analysis of the levels of insulin secretion was calculated on the basis of the insulin released per rudiment.

Determination of protein and insulin synthesis. To measure general protein synthesis, the rudiments were first thawed

and sonicated in 200 μl of distilled water. Five-microliter aliquots were spotted on Whatman 3MM filter paper, washed in 5% TCA followed by 95% ethanol and, finally, by ether. The radioactivity was determined by scintillation spectrometry. To measure insulin synthesis, free [^{3}H]leucine was eliminated from the remainder of the sonicate by repeated precipitation of the protein with 5% TCA. Insulin was then extracted from the pellet according to a micromodification of the Davoren procedure (16) as described by Clark and Rutter (11). The partially purified insulin was applied to the top of a 10.5% polyacrylamide gel and subjected to electrophoresis in a Tris–glycine buffer (pH 8.4) containing 6.5 *M* urea according to Davis (14) and Ornstein (40). When the marker dye (bromophenol) migrated to the bottom of the gel, the gels were removed and sliced at 0.8-mm intervals. Each slice was dissolved overnight at 60°C in 10 ml of a toluene-based scintillation fluid containing 4 g/liter of Omnifluor and 25 ml/liter of NCS solubilizer (Amersham/Searle) and then counted. The insulin peaks were identified by comparison with the electrophoretic mobility of purified insulin and by running pancreatic extracts in which insulin was removed by pretreatment with insulin antibodies before electrophoresis on similar gels (11).

Morphological analysis. The pancreases were fixed with 2% glutaraldehyde in 0.04 *M* sodium phosphate buffer at pH 7.4. After 24 hr, the tissues were washed in 0.132 *M* sodium phosphate buffer, pH 7.4, and postifixed 1 hr at 4°C in 2% osmium tetroxide in the same buffer. The dehydration and embedding were carried out according to the method described by Luft (33). Light microscopic observations were done on Epon-embedded thick sections after methylene-blue azure staining. Thin sections were cut with diamond knives and collected on 75-mesh grids covered by a Parlodion film stabilized with carbon coating. The sections were treated with uranyl acetate followed by lead citrate (52) to increase the contrast. Observations and photographic recordings were then made with a Philips 300 electron microscope.

RESULTS

Pancreatic Growth and Insulin Accumulation in Vitro

The accumulation profiles of insulin, protein and DNA *in vitro* in the developing rat pancreas are shown in Fig. 1. Pancreatic cells divide actively during the first days of cultivation. As differentiation pro-

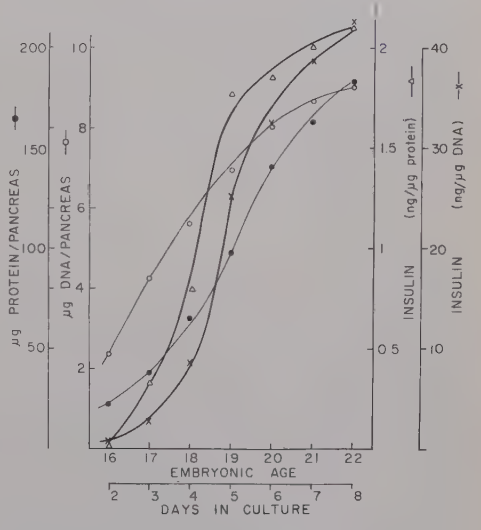


FIG. 1. Accumulation of insulin, protein and DNA in developing pancreas. Day-14 pancreatic rudiments were cultured for 2–8 days as described in Materials and Methods. On the appropriate day the rudiments were harvested, rinsed with EBSS and stored at −70°C. Protein, DNA and insulin were measured as described in Materials and Methods. Solid circles represent protein accumulation; open circles represent DNA accumulation; crosses represent the specific activity of insulin expressed in nanograms per microgram of DNA and the open triangles represent the specific activity of insulin in nanograms per microgram of protein. Each point represents the mean of at least five determinations. The variance analysis after log-transformation shows an experimental error of 0.017 (df = 28) for protein, 0.026 (df = 28) for DNA and 0.10 (df = 48) for insulin content when expressed as nanograms per rudiment.

ceeds, there is a decrease in the extent of cell division as evidenced by the declining rate of DNA accumulation. The rapid increase in protein content and the change in the DNA/protein ratio is the consequence of the accumulation of large amounts of exocrine enzymes in the acinar cells. This leads to a lower estimate of the magnitude of the increase in insulin-specific concentration when normalized against protein. Therefore, throughout this paper the insulin level is normalized against DNA rather than protein. Before day 16, the pancreas rudiments contain less than 1% of the total insulin accumulated by day 22. Between days 16 and 19, insulin accumulates logarithmically with a doubling time of about 10 hr. The specific insulin concentration at the end of the culture period (day 14 plus 8 days of culture) approaches the *in vivo* level at birth (days 21–22 of gestation) (47).

Response of Fetal B Cells to Glucose Stimuli

As shown in Fig. 2, the response of the fetal rudiment to high (16.5 m*M*) glucose (physiological level = 5.5 m*M*) varies throughout the developmental period. After 2 days of culture (equivalent to day 16 of development), high glucose does not stimulate insulin release during a 2-hr incubation. During the next 4 days, however, this level of glucose does stimulate insulin secretion. These amounts of insulin released in the presence of glucose are a relatively constant proportion (about 5%) of the total content of the pancreas. Figure 2 also shows that there is a decreased response of insulin secretion to 16.5 m*M* glucose if pancreases have been cultured for the last 24 hr in a poor nutrient medium (BME, 1× amino acids, 2.75 m*M* glucose). The results of other experiments suggest that there may be less insulin synthesized over a long period of time in amino acid-poor medium;[5] thus, the lower insulin release might simply be related to a decreased total insulin content. This possibil-

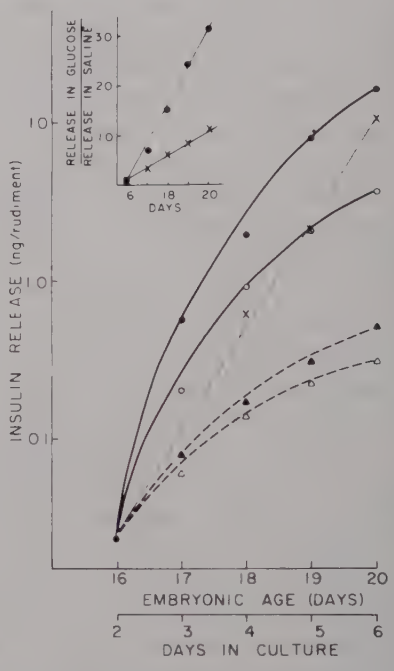


FIG. 2. Effect of the nutritional quality of the medium on the secretion of insulin by cultured pancreatic rudiments. Day-14 pancreatic rudiments were cultured 1–5 days in modified BME (7× amino acids, supplemented with 5.5 m*M* glucose) as described in Materials and Methods. During the last 24 hr of incubation, the medium of half of the rudiments was replaced by the same medium (closed symbols) while the medium of the other half was replaced by BME (1× amino acids, 2.75 m*M* glucose) (open symbols). At the end of the culture period the capacity of both sets of pancreases to secrete insulin in response to 16.5 m*M* glucose was tested. The circles represent the level of insulin released in the presence of 16.5 m*M* glucose and the triangles the basal level for each of the previous culture conditions. An analysis of variance shows that the experimental error is 0.04 (df = 20) in BME, 1× amino acids, 2.75 m*M* glucose and 0.03 (df = 20) in BME, 7× amino acids, 5.5 m*M* glucose. The thin line indicates the theoretical values of insulin secretion expected if the decrease were the sole consequence of a block of insulin accumulation during the last 24 hr and assuming that the percentage of the total insulin content that is released does not change. The insert shows the ratios of the release in the presence of 16.5 m*M* glucose over the release in the absence of glucose in pancreases cultured in BME, 7× amino acids, 5.5 m*M* glucose (closed circles) and those transferred to BME, 1× amino acids, 2.75 m*M* glucose for the last 24 hr (crosses), respectively.

[5] L. Rall, B. Walther, M. de Gasparo, and R. Pictet, unpublished observations.

ty can be effectively eliminated by the following considerations: If the proportion of the pancreatic insulin released remains constant (about 5%) and there were no accumulation of insulin during the last 24 hr in the presence of low levels of amino acids, then the insulin released in response to a glucose challenge should follow a simple logarithmic curve (thin line, Fig. 2). This is in contrast to the results shown. Lowering the amino acid content of the medium decreases the basal as well as the stimulated secretion. The insert in Fig. 2 shows that the ratios of high glucose-stimulated insulin released over the corresponding control are linear throughout the developmental period studied, although the slopes are different for the two nutrient conditions. Thus, some other controlling aspect of insulin secretion is influenced by the altered nutrient conditions.

The inclusion of a physiological level of amino acids (concentrations equivalent to BME) during the 2-hr incubation neither induces a higher basal rate of insulin release nor potentiates the stimulation observed with 16.5 m*M* glucose (Table 1). A physiological level of glucose (5.5 m*M*) also induces insulin secretion on days 18 and 20 but proportionately less on the latter day. Similarly, in culture using BME, 7× amino acids and 5.5 m*M* glucose, the pancreases secrete an increased amount of insulin into the culture medium. The amount secreted is the same decreasing proportion of the total content (3.7 and 1.5% per 6 hr at days 18 and 20, respectively). Thus, amino acids by themselves do not appear to be secretagogues in this system.

Effect of Caffeine on Glucose and Glucagon-Stimulated Insulin Release

Caffeine, an inhibitor of phosphodiesterase, was chosen because of its known effect in potentiating insulin secretion. The concentration of caffeine employed in these experiments was determined from a dose–response curve run in the presence of 16.5 m*M* glucose. The maximum effect on insulin release is attained at 5×10^{-3} *M*, in agreement with a previous report (28). The high level required for maximum effect may be related to a low cellular permeability for this compound but has no detectable side effects during the time of the experiments. Glucagon was added at 10^{-6} *M* since it shows a maximal effect on islet adenylate cyclase at this concentration (15).

TABLE 1

EFFECT OF AMINO ACIDS ON GLUCOSE-STIMULATED INSULIN SECRETION[a]

Additions		Corresponding gestational age			
		18 Days		20 Days	
Amino acids (BME 1×)	Glucose (m*M*)	Insulin release (ng/μg of DNA)	Percent of Total insulin content	Insulin release (ng/μg of DNA)	Percent of Total insulin content
—	—	0.03	0.34	0.06	0.2
+	—	0.03	0.34	0.06	0.2
—	16.5	0.4	4.5	2.0	6.0
+	5.5	0.16	1.8	0.20	0.6
+	16.5	0.53	6.0	2.05	6.4

[a] Pancreatic day-14 rudiments were cultured for 4 or 6 days and stimulated as described in Materials and Methods. The incubation medium contained the levels of amino acids equivalent to BME. The values are averages of at least six experiments. Glucose, 16.5 m*M*, is more effective than 5.5 mM glucose in augmenting insulin secretion both on day 18 (t = 19.23, df = 14) and on day 20 (t = 4.5, df = 15).

Insulin secretion elicited by glucose and glucose + glucagon is dramatically potentiated by caffeine. This effect is displayed in Fig. 3, plotted as a function of developmental age. The basal level of insulin secretion increases throughout development, but it represents a decreasing proportion of the total insulin content (from 1.3% at day 16 to 0.2% at day 20).

In the presence of both caffeine (10 m*M*), and glucose (16.5 m*M*), the amount of insulin released is eight to nine times greater with glucose alone (cf. Fig. 2). Under these conditions, 40–45% of the total hormone content of the pancreas is

secreted during a 2-hr incubation, even on day 16 when glucose alone is ineffective (Fig. 2). The addition of 1 μM glucagon increases this value by 5–10%. As shown in Table 2, caffeine or glucagon alone exerts a small stimulation on insulin secretion consistently throughout development. Their combined effects are additive but not as great as the increment observed with high glucose (16.5 mM) alone (cf. Fig. 2 and Table 2), except on day 16 when glucose by itself exerts no secretory stimulus.

The pattern of response of the day-16 pancreas to various secretagogues is summarized in Table 3. As previously shown in Fig. 2, glucose alone does not stimulate insulin secretion at this stage. However, after addition of glucagon or caffeine, it is effective, and the ratio of the insulin released to the total gland content in response to these agents is highest on this day (compare Table 2). The dramatic effect of high levels of glucose in the presence of caffeine is probably not simply due to an energy requirement supplied by glucose since the addition of lower levels of glucose (2.75 mM), within the physiological range, does not appreciably enhance the caffeine effect.

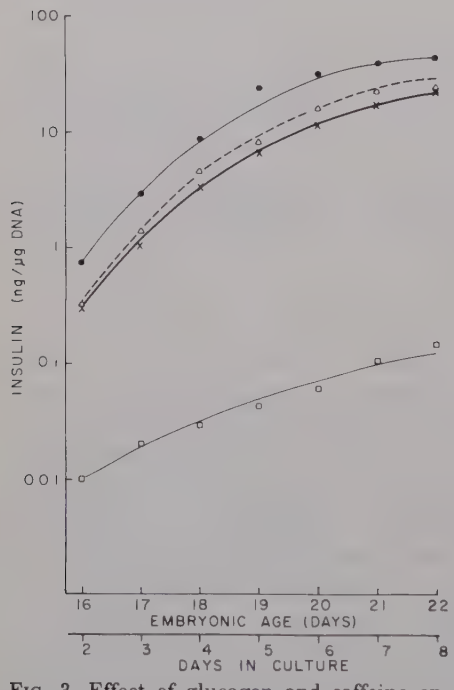


FIG. 3. Effect of glucagon and caffeine on glucose-induced insulin secretion. The effects of the secretagogues were tested on cultured day-14 pancreatic rudiments as described in Materials and Methods and the results are expressed as nanograms of insulin per microgram of DNA. The solid circles represent the pancreatic insulin content; the open squares represent insulin release in the control (saline) solution; the crosses, insulin release in the presence of 10 mM caffeine and 16.5 mM glucose; and the open triangles, insulin release in 10 mM caffeine, 16.5 mM glucose and 1 μM glucagon. The analysis of variance of the insulin release shows an experimental error of 0.06 (df = 43) for the control, 0.14 (df = 47) for glucose + caffeine and 0.09 (df = 47) for glucose + caffeine + glucagon.

Cyclic AMP and Insulin Release

To ascertain whether glucose as well as other insulin secretagogues act through the cAMP system, a comparison was made between the maximal secretory response obtained with glucose and other compounds known to enhance cAMP levels. As shown in Table 4, caffeine plus activators of adenylate cyclase (cholera toxin and glucagon) or Bt_2 cAMP were less effective than glucose alone in the stimulation of insulin secretion by 20-day-old pancreases. Caffeine and glucagon together produced about a tenfold increment over basal as well as glucose-stimulated insulin release. Similarly, glucose produces an approximately 30-fold increment of secretion over basal, and caffeine–glucagon-stimulated values.

Rate of Insulin Secretion during Prolonged Stimulation with Glucose in the Presence of Caffeine and Glucagon

Figure 4 shows that the rate of hormone secretion has reached its maximum within 1 hr. This is analogous to the "second phase" observed in kinetic studies of insulin release from adult (22) and fetal pancreases (30). During the next hour the rate decreases by about 25% and remains approximately constant over the remainder

TABLE 2

EFFECT OF CAFFEINE AND GLUCAGON ON INSULIN SECRETION[a]

Corresponding gestational age (days)	Control		Caffeine (10^{-2} *M*)		Glucagon (10^{-6} *M*)		Caffeine (10^{-2} *M*) + glucagon (10^{-6} *M*)	
	Insulin release (ng/μg of DNA)	Percent of total insulin content	Insulin release (ng/μg of DNA)	Percent of Total insulin content	Insulin release (ng/μg of DNA)	Percent of Total insulin content	Insulin release (ng/μg of DNA)	Percent of total insulin content
16	0.01	1.34	0.049	6.5	0.037	4.8	—	—
18	0.03	0.34	0.15	1.7	0.05	0.5	0.2	2.2
20	0.06	0.20	0.19	0.6	0.10	0.3	0.58	1.8

[a] Day-14 rudiments were cultured for 2, 4 and 6 days and then the effects of the two secretagogues were measured in the absence of glucose, as described in Materials and Methods. The data are the averages of at least five experiments. The secretion induced by glucagon or caffeine over the control is statistically significant on each day; at day 16, $t = 10.28$, $df = 13$ and $t = 9.94$, $df = 13$; at day 18, $t = 26.1$, $df = 14$ and $t = 7.75$, $df = 14$; at day 20, $t = 10.43$, $df = 13$ and $t = 3.07$, $df = 13$ for caffeine and glucagon, respectively; and at day 20, $t = 61$, $df = 13$ for the caffeine–glucagon combination.

TABLE 3

SECRETION OF INSULIN BY DAY-16 PANCREATIC RUDIMENTS

Secretagogues	Concentration (m*M*)	Insulin secretion		
		Nanograms/rudiment	Nanograms/microgram of DNA	Percent of total insulin content
None	—	0.024	0.010	1.3
Glucose	16.5	0.026	0.011	1.4
Glucagon	0.001	0.087	0.037	4.9
Caffeine	10	0.117	0.049	6.5
Glucose	2.75	0.250	0.105	14.0
Caffeine	10			
Glucose	16.5	0.720	0.304	40.0
Caffeine	10			
Glucose	16.5			
Glucagon	0.001	0.75	0.316	42.0
Caffeine	10			

[a] Day-14 pancreases were cultivated for 2 days and incubated in the presence of the various secretagogues and insulin secretion was measured as described in Materials and Methods. The data are averages of at least six experiments. The statistical significance has been analyzed by comparison with the control (saline) values. Glucose alone had no significant effect, while the others produced significant stimulations of insulin release. The Student's *t* test already shows a significance between glucagon and control ($t = 9.94$, $df = 13$).

of the 4-hr experimental period. These results indicate that the pancreas is in good functional condition for periods longer than those used in this study. The tissues also appear normal morphologically, even after 6 hr of incubation (Figs. 5 and 6).

Increased Insulin Synthesis after Stimulation of Insulin Secretion

The rate of insulin synthesis was measured in day-18 and -20 pancreases before and after stimulation (see Table 5). There was no significant difference in total protein synthesis between stimulated and unstimulated pancreases. However, there was a twofold increase in the rate of insulin synthesis relative to total protein synthesis over a 24-hr period in pancreases incubated under conditions in which 50% of the total insulin has been secreted. The kinetics of insulin synthesis, however, differ from that of total protein. The incorporation of the amino acid into total protein is linear following stimulation, but there is a lag of approximately 6 hr in both control and stimulated tissues before the synthesis of insulin is measurable. After the lag, synthesis proceeds linearly. In other experiments, a shorter lag has been reported (42). Our results imply that some aspect of our present conditions critically but only temporarily limits synthesis of insulin but not of total protein.

The ultrastructure of islets was exam-

TABLE 4

SYNERGISTIC EFFECTS OF COMBINATION OF SECRETAGOGUES ON INSULIN RELEASE IN PANCREATIC RUDIMENTS[a]

Added secretagogues	Insulin release (ng/μg of DNA)			
	Saline	Bt_2 cAMP	Cholera toxin	Glucagon
Saline	0.06	0.11	0.08	0.10
Caffeine	0.19	0.45	0.51	0.58
Glucose	2.01	2.80	4.30	7.60
Glucose + caffeine	11.10	14.80	15.80	15.60

[a] Day 14-rudiments were cultured for 6 days and tested as described in Materials and Methods. The effects of 10^{-6} *M* cholera toxin, glucagon or Bt_2cAMP on the 16.5 m*M* glucose-induced, 10 m*M* caffeine-induced or 16.5 m*M* glucose plus 10 m*M* caffeine-induced secretion of insulin were compared. The *t* test was performed on the insulin values expressed as nanograms per rudiment. In the absence of glucose or caffeine, there is a statistically but certainly not biologically significant effect of Bt_2cAMP and glucagon alone but not of cholera toxin. On the other hand, each of these three compounds potentiate caffeine-induced insulin secretion to the same extent ($t = 2.352$, $df = 32$). Glucagon, cholera toxin and Bt_2cAMP potentiate glucose-induced secretion as well. However, glucagon ($t = 6.72$, $df = 10$) is more effective than cholera toxin ($t = 5.30$, $df = 10$) which in turn is more effective than Bt_2cAMP ($t = 3.67$, $df = 12$). In the presence of glucose and caffeine together, the three compounds produce a significant increment over the control value ($t = 2.03$, $df = 32$), but there is no difference in the magnitude of their effects.

ined following 2-hr incubation under control (Fig. 7) or maximal (Fig. 8) stimulation. Since the rapid accumulation of insulin between 18 and 20 days of fetal development (see Fig. 1) is accompanied by a great variation in the number of β granules in individual B cells (Fig. 7), a change in the content of β granules even by 50% is difficult to demonstrate in electron micrographs. However, many B cells in the stimulated pancreases show a great accumulation of β granules within the Golgi apparatus (Fig. 9). This condition is rarely observed in control pancreases.

DISCUSSION

The embryonic rat pancreas develops normally *in vitro* as indicated by a several-fold increase in cell number and a large increase in specific products including insulin. Furthermore, ultrastructural studies show that the various cytoplasmic organelles, including microtubules and microfilaments which may be required for secretion, are present in B as well as other pancreatic cells *in vitro* (45, 46). Therefore this system is suitable for our studies on insulin secretion.

The insulin concentration of cultured pancreases approaches the values found in fetuses of corresponding ages (47). By day 16, *in vitro* or *in vivo*, the low rate of insulin synthesis characteristic of the prodifferentiated state has increased (47) and one observes the first β granules within the B cells (46, 47). However the pancreas has accumulated no more than 1% of the total insulin content found in the differentiated pancreas. Even at this early stage, we have shown that insulin secretion is detectable and can be modulated by various secretagogues, including glucagon which is present at differentiated levels within A cells of the pancreatic rudiment throughout this transition (47).

The integrity of the B cells following stimulation with glucagon, glucose and caffeine is preserved as indicated by their normal morphological appearance. In addition insulin is synthesized at an enhanced rate. The latter observation suggests that insulin synthesis may at least in part be regulated by secretagogues or by the secretory process. Permutt and Kipnis (42) as well as others have reported a significant increase in insulin synthesis following a secretory challenge in isolated adult (31, 42, 43, 57, 61) or newborn (58) islets. This increase appears to be regulated both at the transcriptional and translational levels (42). In our system, the increase lasts for many hours after the removal of the inducing factor; therefore, the enhancement of insulin synthesis results from but is not temporally coupled to the secretory stimulus. This increased insulin synthesis following secretion is even observed in pancreases during the developmental period

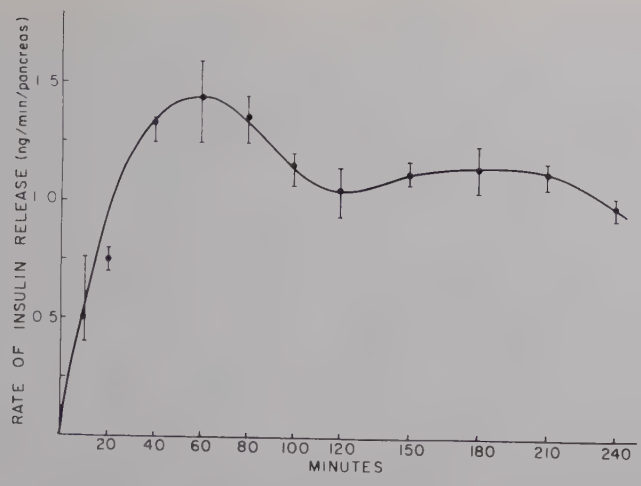


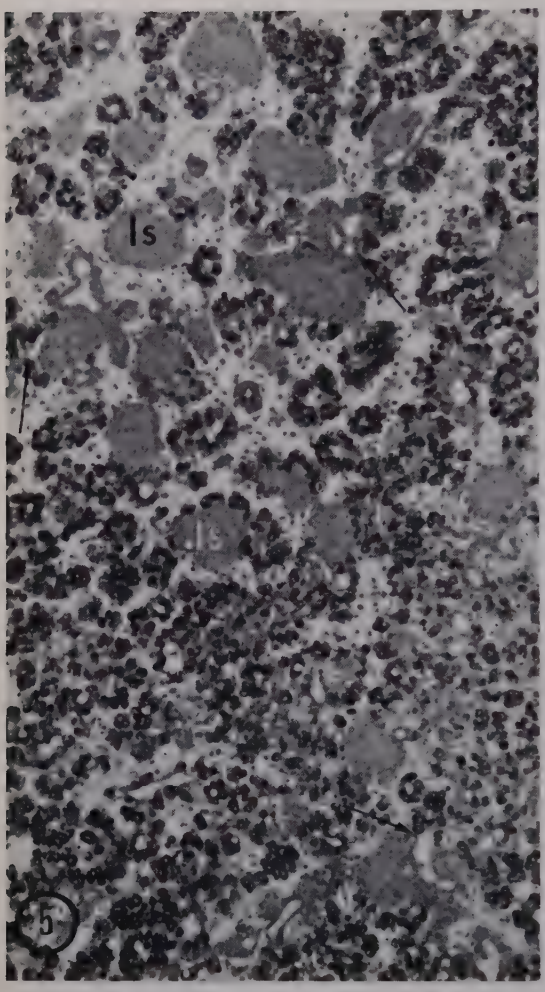
FIG. 4. Insulin release by pancreatic rudiments during prolonged stimulation. Day-14 rudiments were cultured for 6 days and incubated with glucose (16.5 m*M*), caffeine (10 m*M*) and glucagon (1 μM) as described in Materials and Methods. The incubation medium was collected for insulin assay and replaced with fresh medium containing the same stimulants at 0, 10, 20, 40 and 60 min for the first hour, every 20 min for the second hour and every 30 min for the third and fourth hours. Each point represents the mean and the range of three experiments.

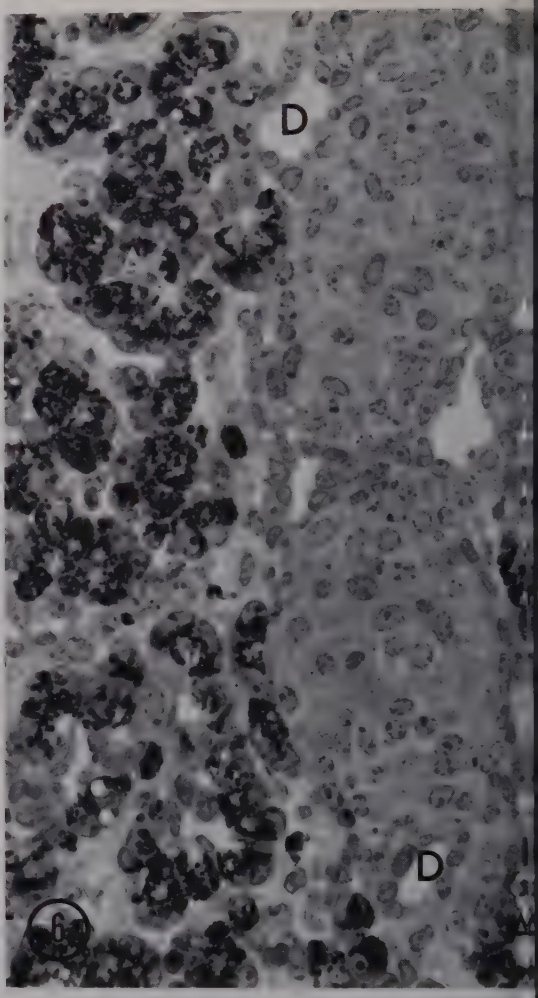
when insulin synthesis is maximal (18 days) (45, 48). This implies that the time course of B cell differentiation may be regulated but it is not limited by the rate of insulin synthesis.

Some reports indicate that amino acids act as secretagogues in adult (35–37) and in fetal islets (27, 28). In our system, the basal level of insulin secretion is not affected by the addition of a mixture of amino acids to the physiological salt medium. On the other hand, the nutritional quality of the medium in which the rudiments are first cultured influences the synthesis of insulin and also the effectiveness of the secretagogues tested. The nutritional environment can therefore conceivably influence the availability of the hormone.

The characteristics of the secretory system are somewhat different in the early and mature pancreas. Glucose alone does not induce secretion in the day-16 pancreas. A glucose level-sensing mechanism, however, must be present since caffeine induces insulin release in proportion to the amount of glucose present. During the following days the B cells become sensitive to glucose alone; on both day 18 and 20, 5.5 m*M* glucose stimulates the release of a significant amount of insulin. Stimulation with 16.5 m*M* glucose results in the secretion of an increasing amount of insulin with age, corresponding to a similar percentage (5–6%) of the total content on day 18 or 20. Treatment with the combined optimal levels of glucose, glucagon and caffeine produces a secretory response also corresponding to a constant proportion (about 50%) of the insulin content over the entire developmental period. Thus, the amount of insulin released, under these defined conditions, may be a function of the B cell content.

Our observations demonstrate that the secretory competence develops simultaneously with the appearance of the storage form of the hormone. The finding that glucose stimulates the secretion of insulin in our pancreatic rudiments, contrasts with our studies in which pancreases explanted later in development (18 days) fail to secrete insulin when stimulated with high levels of glucose (1, 2, 6, 18, 26, 28, 29, 59, 62, 63, 68). It is reported that this capacity to respond to glucose develops shortly after birth but is absent if pancreases are explanted at 18 days and allowed to develop





FIGS. 5 and 6. Cultured pancreases are morphologically normal following stimulation of insulin secretion. Day-14 pancreases were cultured 6 days and then incubated for 6 hr in the presence of 10 m*M* caffeine, 16.5 m*M* glucose and either 10^{-6} *M* Bt_2cAMP (Fig. 5) or 10^{-6} *M* glucagon (Fig. 6). Both exocrine and endocrine tissues are well preserved. The intercellular space is increased during incubation, and the islets become somewhat separated from the exocrine tissue as *in vivo*. At this stage of development the islets are still associated with the exocrine duct (arrows). The acinar lumen are empty, thus there is not an appreciable release of enzymes, an indication that the acinar cells do not respond to the secretory stimuli. Islets (Is), Acini (Ac), ducts (D); Fig. 5, × 115; Fig. 6, × 450.

in vitro over the equivalent period of time (6, 29). This result suggests that an extrapancreatic stimulus is required for the appearance of glucose sensitivity. The apparent discrepancy between our results and the previous ones may originate from the experimental technique. Pancreases at the end of their development (18 days and later) are difficult to dissect and handle without mechanical damage. Since the exocrine cells have by this time accumulated high levels of exocrine enzymes, the destruction of some cells would result in the release of proteolytic enzymes that would be deleterious for both, cells and secreted insulin. On the other hand, cultivation of the pancreatic rudiments *in vitro* may result in the precocious development of glucose sensitivity. This would be the first example in the pancreas of a function de-

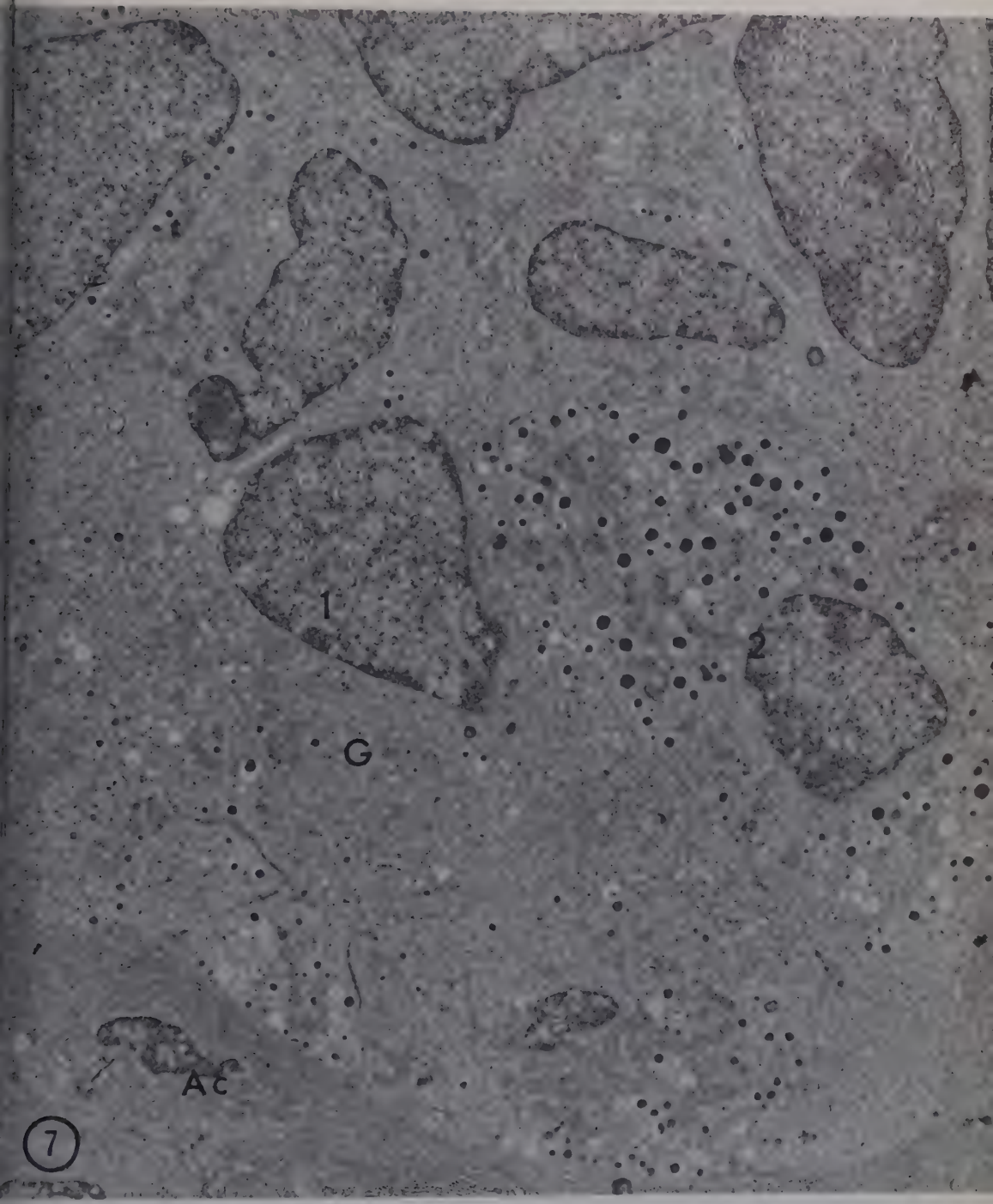


FIG. 7. Ultrastructural appearance of B cells after incubation. Day-14 pancreases were cultured 4 days and then incubated 2 hr in the presence of 10 m*M* caffeine. The ultrastructural integrity of the B cells is preserved. There is no swelling of the endoplasmic reticulum or the mitochondria. The cells show a great variability in the number of β granules (cf. cells labeled 1 and 2). Golgi apparatus (G), acinar cells (Ac). × 7000.

TABLE 5

INSULIN AND PROTEIN SYNTHESIS IN PANCREATIC RUDIMENTS FOLLOWING INSULIN RELEASE[a]

Incorporation	Time of incorporation (hr)							
	2		12		18		24	
	Control trol	Stimulated	Control	Stimulated	Control	Stimulated	Control	Stimulated
[^{3}H]leucine incorporation into total protein ($\times 10^3$) (dpm)	735	690	3,413	3,486	4,945	5,385	6,072	6,116
[^{3}H]leucine incorporation into insulin (dpm)	—	—	5,217	9,450	14,023	27,849	25,209	41,553

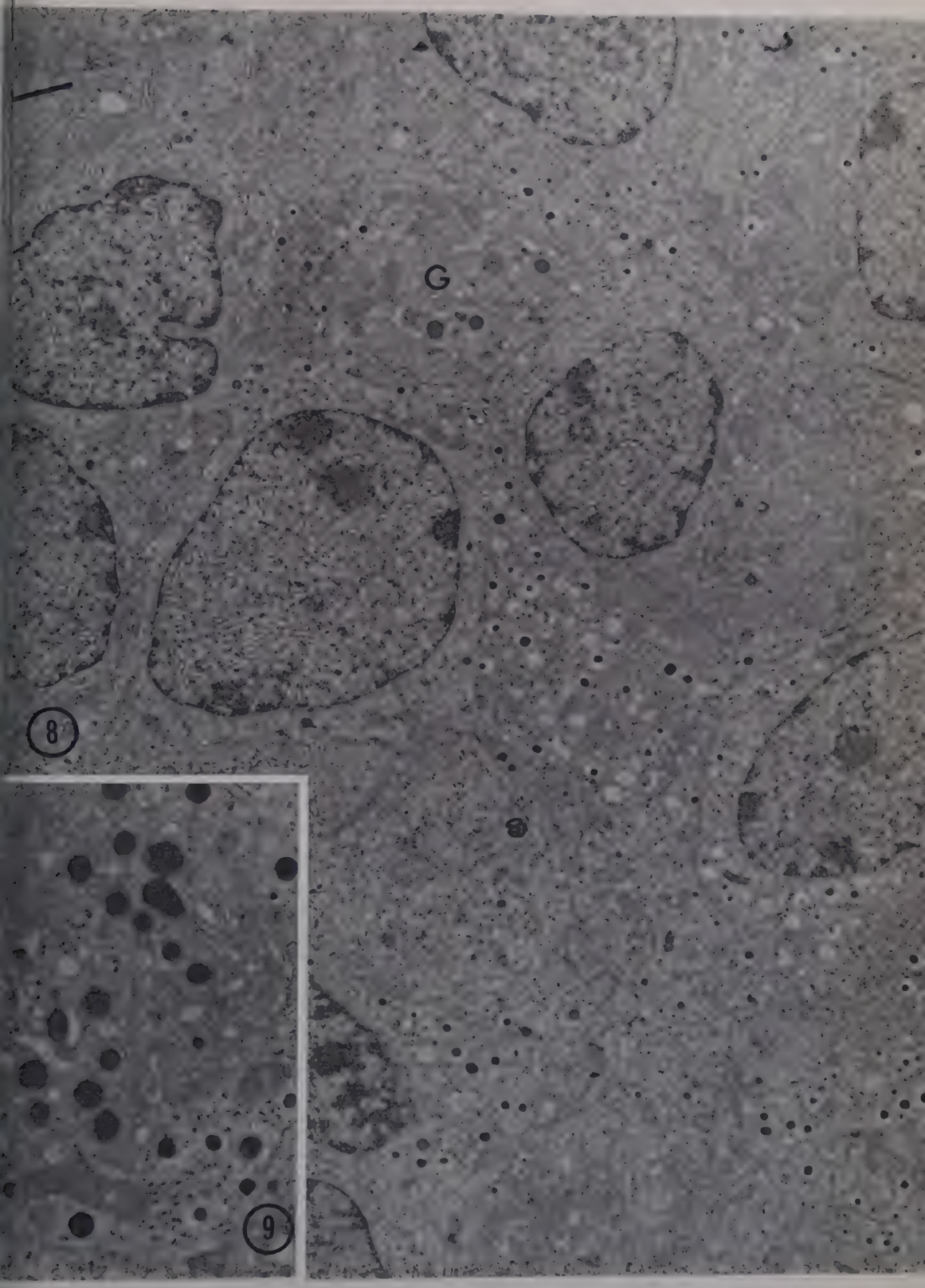
[a] Day-14 pancreses were cultured 4 days and then incubated for a 2-hr period with glucose and caffeine as described in Materials and Methods. After this stimulation period, the incubation medium was assayed for insulin. The pancretic rudiments were washed and placed in culture in the presence of [^{3}H]leucine (20 μCi/ml; 5 Ci/mmol) in 7 × BME, a medium condition which saturates the intracellular leucine pool (24 After 2, 12, 18 and 24 hr, sets of three rudiments were harvested. Insulin and total protein synthesis were determined as described in the Materials and Methods. Results are expressed as disintegrations per minute per rudiment.

veloping at a different pace *in vivo* and *in vitro*. Asplund (3) has reported that repeated infusion of a mother with glucose induces sensitivity to this secretagogue in the fetal pancreas, and Kervran and Girard (25) also observed that even a single 1-hr period of glucose infusion in the mother caused an increase of insulin in the fetal blood. This response after 1 hr is compatible with the view that these pancreases, *in vivo* as *in vitro*, are sensitive to glucose and supports our observations.

A previous report on the levels of circulating insulin in fetuses (19) suggests that the prenatal pancreas secretes insulin *in vivo* in physiological concentrations. However, this insulin may not affect all potential target organs. Felix *et al.* (20) have shown that the fetal diaphragm muscle lacks insulin sensitivity, presumably because of the absence of insulin receptors. Thus, the physiological role of insulin could be restricted in the fetus to some specific target cells like brown adipose tissue which develops earlier than white fat cells (44) and liver; indeed, the ability of B cells to secrete insulin parallels the accumulation pattern of glycogen in the liver (4). In fact, glycogen synthesis may depend upon the presence of insulin (17). Insulin also plays a role in the induction of tyrosine aminotransferase in fetal liver (34) and in the *in vitro* development of the mammary gland (60, 64, 65).

The dramatic effect of caffeine on the glucose response has led to the postulate that there is a high phosphodiesterase activity in the fetal B cells (6, 10, 29, 51) and

FIGS. 8 and 9. Ultrastructural appearance of islet cells after stimulation of insulin secretion. Day-14 pancreases were cultured 4 days and then incubated 2 hr in the presence of 10 m*M* caffeine, 16.5 m*M* glucose and 10^{-6} *M* glucagon. The islet cells are closely associated with duct cells (arrow), a common feature of fetal pancreases. The amount of granules present in differentiated B cells (in adult) varies considerably from cell to cell. In a fetus at that stage of development there is an additional cause of variation since insulin accumulates rapidly. There is considerble variation in the number of granules in the various B cells since their differentiation is not synchronized (45). It is thus difficult to demonstrate conclusively a decrease in the β granule content following secretion without an extensive analysis. Comparison of Figs. 7 and 8 emphasizes this feature. A striking feature is the unusually large number of β granules present in the Golgi apparatus of many B cells in stimulated pancreases (Fig. 9). This probably reflects increased insulin synthesis. The concentration of granules in the Golgi apparatus occurs in the absence of extracellular amino acids. Golgi (G); Fig. 8, × 7000; Fig. 9, × 13,000.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8
9

that the effect of caffeine is due to enhanced cAMP produced by its inhibition of phosphodiesterase activity (53). This would contrast with the situation reported in other tissues; Hommes and Beere have found little difference in the phosphodiesterase levels in a number of tissues from embryonic, fetal and adult animals (23). If caffeine acted via phosphodiesterase, one might expect a weak response to adenylate cyclase stimulants, since the additional cAMP produced would be hydrolyzed rapidly by the high levels of phosphodiesterase. In contrast, we find that glucagon alone is almost as efficient as caffeine alone. Thus, glucagon should be approximately as effective as caffeine in potentiating glucose-stimulated insulin secretion; we find in this case that caffeine is much more effective than glucagon. Therefore, it seems possible that caffeine may potentiate the glucose response by another mechanism in addition to its inhibition of phosphodiesterase. For example, caffeine may affect calcium ion mobilization (39, 49, 50, 66, 67). It has been reported that the caffeine analog theophylline acts on cellular calcium distribution in islets (5), and calcium is known to be required for normal insulin release (13, 21).

It is clear that cAMP plays a key regulatory role in secretion, but how cAMP and glucose interact to regulate secretion is still an open question. Charles *et al.* (8, 9) have observed in adult islets that the addition of theophylline produces high cAMP levels than glucose, but theophylline is much less efficient in stimulating insulin release. Thus the glucose effect cannot be mediated solely through cAMP. We report here that glucose alone in the 20-day embryonic pancreas is one order of magnitude more efficient than caffeine and fourfold more than caffeine combined with either glucagon, cholera toxin or Bt_2cAMP. Thus, optimal levels of combinations of various compounds known to stimulate adenylate cyclase and depress phosphodiesterase are less effective than glucose alone. The basal and glucose-induced levels of secretion are both increased by a factor of about ten by the simultaneous stimulation of adenyl cyclase and by caffeine. Thus, glucose and caffeine appear to act independently and synergistically, and cAMP is apparently not the sole mediator for glucose action in insulin release.

The results of these studies indicate that, in B cells, secretory competence is correlated with the accumulation of the cell-specific product, insulin in β granules. Therefore, packaging and release of the hormone is regulated as a single or coordinated mechanism. The different sensitivity to secretagogues, particularly glucose, existing between fetuses and postnatal animals probably is an adaptation of the mechanism for regulating secretion. The evidence suggests the glucose-induced secretion may not be mediated solely via cAMP. The number of glucose receptors or the mechanism by which they induce secretion may be altered by birth. During fetal development there are likely specific functions for insulin. At birth there may be an additional function associated with the utilization of food. This additional mechanism regulating insulin secretion may be linked to that for regulating exocrine secretion which also develops at birth. Presumably these changes are under the influence of specific hormones and are independent of the fundamental mechanisms regulating pancreatic differentiation.

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Isolation of Myosin-Synthesizing Polysomes from Cultures of Embryonic Chicken Myoblasts before Fusion[1]

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Mononucleated myoblasts and multinucleated myotubes were obtained by culturing embryonic chicken skeletal muscle cells. Comparison of total polysomes isolated from these mononucleated and multinucleated cell cultures by density gradient centrifugation and electron microscopy revealed that mononucleated myoblasts contain polysomes similar to those contained by multinucleated myotubes and large enough to synthesize the 200,000-dalton subunit of myosin. When placed in an *in vitro* protein-synthesizing assay containing [^{3}H]leucine, total polysomes from both mononucleated and multinucleated myogenic cultures were active in synthesizing polypeptides indistinguishable from myosin heavy chains as detected by measurement of radioactivity in slices through the myosin band on sodium dodecyl sulfate (SDS)–polyacrylamide gels. Fractionation of total polysomes on sucrose density gradients showed that myosin-synthesizing polysomes from mononucleated myoblasts may be slightly smaller than myosin-synthesizing polysomes from myotubes. Multinucleated myotubes contain approximately two times more myosin-synthesizing polysomes per unit of DNA than mononucleated myoblasts, and the proportion of total polysomes constituted by myosin polysomes is only 1.2 times higher in multinucleated myotubes than it is in mononucleated myoblasts. The results of this study suggest that mononucleated myoblasts contain significant amounts of myosin messenger RNA before the burst of myosin synthesis that accompanies muscle differentiation and that a portion of this messenger RNA is associated with ribosomes to form polysomes that will actively translate myosin heavy chains in an *in vitro* protein-synthesizing assay.

INTRODUCTION

Fusion of mononucleated myoblasts into multinucleated myotubes during muscle differentiation is accompanied by extensive alterations in nucleic acid metabolism (Clissold and Cole, 1973; Love *et al.*, 1969; Marchok and Wolff, 1968; O'Neill and Strohman, 1969; Paterson and Strohman, 1972; Scholl *et al.*, 1968; Stockdale, 1970; Thi Man and Cole, 1974), cyclic nucleotide metabolism (Novak *et al.*, 1972; Wahrman *et al.*, 1973b; Zalin and Montague, 1974), energy metabolism (Keller and Nameroff, 1974; Shainberg *et al.*, 1971; Tarikas and Schubert, 1974; Turner *et al.*, 1974; Wahrman *et al.*, 1973a), and membrane functions (Fambrough, 1974; Fambrough and Rash, 1971; Fambrough *et al.*, 1974; Fluck and Strohman, 1973; Hartzell and Fambrough, 1973; Lough *et al.*, 1972; Tennyson *et al.*, 1973; Wilson *et al.*, 1973). In addition, it is clear that rapid synthesis of the 200,000-dalton subunit of myosin begins abruptly 4–8 hr after the onset of fusion (Coleman and Coleman, 1968; Morris *et al.*, 1972; Paterson and Strohman, 1972; Stockdale and O'Neill, 1972; Yaffe and Dym, 1972). The extent to which myosin is synthesized in cultures of mononucleated myoblasts, however, has not been firmly established. Although Coleman and Coleman (1968) and Yaffe and Dym (1972) could detect no myosin heavy chain synthesis in cultures of mononucleated myoblasts, Paterson and Strohman (1972) reported a low level of myosin synthesis in such cultures. Paterson and Strohman, however, attributed this small amount of myosin synthe-

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sis entirely to the few small myotubes that contaminate myoblast cultures. On the other hand, Morris *et al.* (1972) and Stockdale and O'Neill (1972) found low levels of myosin heavy chain synthesis in mononucleated muscle cells before the onset of cell fusion. In addition, Rubenstein *et al.* (1974) have recently detected myosin and actin synthesis in cultures of mononucleated myoblasts as well as in cultures of embryonic fibroblasts and chrondroblasts.

The few studies done thus far on appearance of the messenger RNA coding for the heavy chains of myosin during muscle differentiation have also been conflicting. Results of Yaffe and Dym's experiments with actinomycin D administration at different times during myogenesis (Yaffe and Dym, 1972) suggest that myosin messenger RNA may be present in mononucleated myoblasts; others, in working with extracts of prefusion myoblasts, however, have been unable to isolate a messenger RNA that would direct synthesis of the 200,000-dalton subunit of myosin in a reticulocyte cell-free protein synthesizing assay (Pryzbyla *et al.*, 1973; Pryzbyla and Strohman, 1974; Strohman *et al.*, 1974). Recently, Buckingham *et al.* (1974) identified in cultures of mononucleated fetal calf muscle cells a 26S polyadenylic acid-containing RNA that hybridized to DNA complementary to myosin messenger RNA with kinetics identical to those of the original myosin messenger RNA. The capacity of this 26S RNA to direct synthesis of myosin heavy chains in a cell-free protein-synthesizing assay was not demonstrated, however. We have analyzed muscle cell cultures at different stages of differentiation for polysomes capable of synthesizing polypeptides that would migrate with the 200,000-dalton subunit of myosin during SDS–polyacrylamide-gel electrophoresis.[3] The results of our study have identified such polysomes both in cultures of mononucleated myoblasts and in multinucleated myotubes.

[3] Abbreviation used: SDS–polyacrylamide-gel electrophoresis is polyacrylamide-gel electrophoresis done in the presence of sodium dodecyl sulfate.

MATERIALS AND METHODS

Preparation of muscle cell cultures. After removal of skin and bones, leg muscle from four to six 12-day-old chicken embryos was suspended in 10 ml of complete medium (85% Eagle's minimum essential medium, 10% horse serum, 5% chicken embryo extract, 50 units of penicillin/ml, 50 μg of streptomycin/ml, and 2.5 μg of Fungizone/ml). The tissue was dissociated with a Vortex mixer at maximum speed for 20 sec and filtered through two sterile Swinnex filters, the first one containing 200 × 200 mesh nylon cloth and the second a double layer of lens paper. The filtrate was centrifuged at 700*g* for 5 min, and the cell pellet was resuspended in complete medium by aspiration. Cells were counted with a hemocytometer and plated in 15-cm Falcon tissue culture dishes (coated with 1.77 μg/cm^2 of collagen) such that cell density after 24 hr of incubation was approximately 1.1×10^4 cells/cm^2. Cultures were incubated at 37°C in an atmosphere of 95% air and 5% CO_2 in 15 ml of complete medium, which was changed every 24 hr.

Nuclei counts. Cultures to be counted were rinsed two times with an isotonic saline solution at 37°C, fixed in absolute methanol for 5 min, and stained with Giemsa stain for 20 min at room temperature. At least 1000 nuclei were counted in randomly chosen fields, and both the total number of nuclei per dish and the percentage of total nuclei within multinucleated fibers were calculated from these data.

Pulse labeling of cultures. The rate of myosin heavy chain synthesis at various stages of differentiation was determined by pulse labeling with [^{3}H]leucine in a manner similar to that of Paterson and Strohman (1972). Cultures in 10-cm dishes were labeled at 37°C for 4.0 hr in 4.0 ml of complete medium containing 10 μCi of [^{3}H]leucine/ml (specific radioactivity 5 Ci/mmol). At the end of the labeling pe

od, the dishes were rinsed twice with cold .25 *M* potassium chloride, 0.02 *M* Tris, pH .4, and the cells were scraped from the urface with a plastic spatula into 1.0 ml of he 0.25 *M* potassium chloride, 0.02 *M* ris, pH 7.4, buffer. Cells were homogeized with 30 strokes of a 7-ml Dounce omogenizer (B pestle), and the homogeate was centrifuged at 12,000*g* for 10 min. nough cold water was added to lower the otassium chloride concentration to 0.025 *M*, the tubes were left at 2°C for 2 hr, and yosin-containing material was pelleted t 12,000*g* for 20 min. The pellet was disolved in 0.075 ml of 1.5 *M* 2-mercaptoethaol, 0.01% bromophenol blue, 1.5% sodium odecyl sulfate, 60 m*M* sodium phosphate, H 7.0, and 6% glycerol by heating at 00°C for 10 min. Samples were then elecrophoresed according to the procedure of Weber and Osborn (1969) on 7.5% polyacrylamide gels. Gels were stained with 0.1% Coomassie blue and destained electrophoretically in a H_2O:methanol:acetic cid mixture (87.5:5:7.5, by volume). Destained gels were frozen in Dry Ice, and a eries of 0.8-mm slices were taken through he region of the gels containing the 200,000-dalton subunit of myosin. Slices were dissolved in 0.2 ml of 30% hydrogen eroxide by heating at 50°C for 3 hr in olyethylene minivials, 4.5 ml of Aquasol New England Nuclear) was added to each ial, and the radioactivity was counted after cooling for at least 24 hr) in a Model 3320 Packard liquid scintillation spectrometer. Counts per minute were converted nto disintegrations per minute by using he automatic external standardization method with either chloroform or pyridine as the quenching agent.

Isolation of polysomes. Three to four hours before polysome isolation, cultures were fed 15 ml of complete medium at 37°C. Cultures were removed from the incubator, and the medium was poured off and very quickly was replaced with approximately 25 ml of ice-cold isolation buffer (0.25 *M* potassium chloride, 0.01 *M* magnesium chloride, 0.01 *M* Tris, pH 7.4) to chill the cultures and to rinse out remaining complete medium. All subsequent steps were performed at 2°C. Cells were scraped from the surface of the dishes into cold isolation buffer containing 0.5% Triton X-100 and were lysed as described by Morse *et al.* (1971). For preparation of total polysomes, the supernatant fluid remaining after centrifugation of the lysate at 12,000*g* for 10 min was layered onto 2.0 ml of isolation buffer containing 1.5 *M* sucrose and was centrifuged in a Beckman Ti 50 rotor at 180,000g_{max} for 2.0 hr. Pelleted polysomes from either fractionated or total polysome preparations were resuspended in 0.25 ml of incubation buffer (0.15 *M* KCl, 5.0 m*M* $MgCl_2$, 6.0 m*M* 2-mercaptoethanol, 10% glycerol, 20 m*M* Tris, pH 7.6), and the resuspended polysomes were analyzed for their capacity to synthesize myosin heavy chains in an *in vitro* assay for protein synthesis (Heywood *et al.*, 1967; Rourke and Heywood, 1972) as described in detail below. Generally, total polysomes from eight to twelve 24-hr cultures and three to six 72-hr cultures were assayed in duplicate for myosin-synthesizing polysomes in a single experiment.

Polysomes from 14-day-old embryonic muscle were prepared according to the procedure of Heywood *et al.* (1967).

Assay of myosin-synthesizing polysomes. Assay of myosin-synthesizing polysomes was performed at 37°C in the presence of 4.0 m*M* ATP, 1.0 m*M* GTP, 6.0 m*M* 2-mercaptoethanol, 10 μM each of 19 unlabeled amino acids, 10 μM [^{3}H]leucine (5 Ci/mmol), 2.6 mg/ml of phosphocreatine, 0.2 mg/ml of creatine phosphokinase, 0.1 mg/ml of transfer RNA prepared from embryonic chicken muscle according to von Ehrenstein (1967), 4.0 mg/ml of crude aminoacyl tRNA synthetases prepared according to Rourke and Heywood (1972), 150 m*M* KCl, 5.0 m*M* $MgCl_2$, 10% glycerol, and 10 m*M* Tris, pH 7.6. Aliquots were removed after various times and were placed into enough cold (2°C) water to

lower the KCl concentration to 25 m*M*. After thorough mixing, tubes were left at 2°C for 2–4 hr, and the myosin-containing material was pelleted at 2000*g* for 40 min [crude aminoacyl tRNA synthetases prepared by the procedure of Rourke and Heywood (1972) contain approximately 15 μg of myosin/mg of synthetases, and this myosin is utilized very effectively as carrier myosin]. The pellet was resuspended, electrophoresed on SDS–polyacrylamide gels, and analyzed for incorporation of radioactivity into myosin heavy chains as described under pulse labeling of cultures. All pipets, centrifuge tubes, and water were autoclaved before use in polysome preparation or amino acid incorporation to reduce ribonuclease contamination.

Electron microscopy of polysomes. Polysomes from the heavier region of the gradients were fixed at 2°C for 1.5 hr in 1.25% glutaraldehyde, 10 m*M* $MgCl_2$, 10 m*M* Tris, pH 7.4, and were pelleted onto carbon-coated grids at 150,000*g* for 1.5 hr. Polysomes were stained for 1.0 min with 2% uranyl acetate and examined by electron microscopy. Concentration of polysomes was determined by measuring the absorbance at 260 nm and using an extinction coefficient of 11.2 OD units/mg of polysomes/ml.

RESULTS AND DISCUSSION

Cultures of embryonic chicken muscle cells have been widely used to study the relationship of myosin synthesis to fusion of mononucleated myoblasts into multinucleated myotubes (Coleman and Coleman, 1968; Morris *et al.*, 1972; Paterson and Strohman, 1972; Stockdale and O'Neill, 1972; Yaffe and Dym, 1972). Under the culure conditions described in Materials and Methods, the percentage of nuclei within myotubes increases from less than 5% after 30 hr in culture to a plateau of 60–70% after 55 hr (Fig. 1). Immediately after this burst of fusion, approximately a sevenfold increase in the rate of myosin heavy chain synthesis per unit of DNA occurs

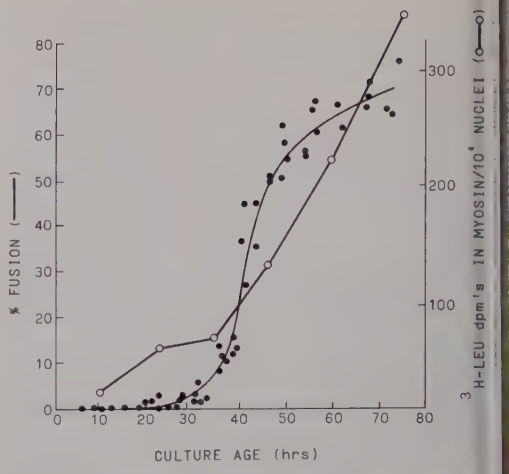

FIG. 1. Kinetics of fusion and myosin synthesis in myogenic cell cultures. Percent fusion and rate of myosin heavy chain synthesis were determined as described in Materials and Methods. Each point is the mean of duplicate determinations.

(Fig. 1). These results are in general agreement with other reports on the kinetics of myoblast fusion and myosin synthesis (Morris *et al.*, 1972; Paterson and Strohman, 1972; Stockdale and O'Neill, 1972).

Heywood *et al.* (1967) have demonstrated that polysomes containing 50–60 ribosomes in embryonic chicken muscle are responsible for synthesis of the 200,000-dalton heavy chain of myosin. Because rate of myosin synthesis per unit of DNA (Fig. 1) increases sevenfold soon after the onset of fusion, cultures of multinucleated myotubes were expected to contain more large 50–60-ribosome polysomes than cultures of mononucleated myoblasts when compared on sucrose density gradients. Polysomes were isolated from 24- and 72-hr cultures of cells that contained similar numbers of nuclei. An optical density tracing at 260 nm of the density gradient separations of the polysome fractions from these 24- and 72-hr cultures is shown in Fig. 2. Although, as expected (Hosick and Strohman, 1971), the 72-hr cultures contained more total polysomes per nucleus than the 24-hr cultures (greater optical density in Fig. 2; also, see left column, Table 3), the distribution of polysome sizes

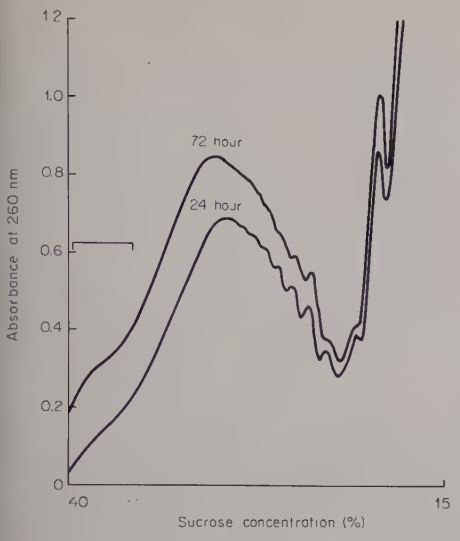

FIG. 2. Polysomes from 24- and 72-hr muscle cell cultures isolated as described in Materials and Methods and displayed on a linear 15–40% sucrose gradient in isolation buffer after centrifugation at 150,000g_{max} for 2.0 hr in a Beckman SW 25.2 rotor. Direction of sedimentation is from right to left. Bracket denotes the part of the gradient from which polysomes were isolated for electron microscopy. Both 24- and 72-hr samples were obtained from cultures containing approximately 7×10^7 nuclei.

from cultures at these two stages of differentiation was qualitatively similar (Fig. 2). The similarity in proportion of polysomes in the bottom one-third of the gradient (at the left side of Fig. 2) is especially interesting because this is the region of the gradient from which myosin-synthesizing polysomes have isolated (Heywood *et al.*, 1967). Polysomes from the region of the gradients designated by the bracket in Fig. 2 were isolated and prepared for electron microscopy as described in Materials and Methods. Figure 3 shows that polysomes in this region of the gradients from both fused and nonfused cultures were large enough to synthesize a peptide chain as large as myosin heavy chains.

The presence of polysomes large enough to support synthesis of the 200,000-dalton subunit of myosin in mononucleated muscle cells prompted an investigation into whether these polysomes would incorporate [^{3}H]leucine into polypeptides that would migrate with the heavy chains of myosin in SDS–polyacrylamide-gel electrophoresis. To demonstrate that the assay described in Materials and Methods would accurately measure synthesis of myosin heavy chains, total polysomes from leg muscle of 14-day-old embryonic chickens were isolated and analyzed for ability to incorporate amino acids into protein. Proteins in the *in vitro* protein-synthesizing system were separated by SDS–polyacrylamide-gel electrophoresis, and, after staining, the SDS–polyacrylamide gels were sliced and the slices assayed for radioactivity, as described in Materials and Methods. Radioactivity in the gel slices increased at the distance of migration corresponding to a molecular weight of 200,000

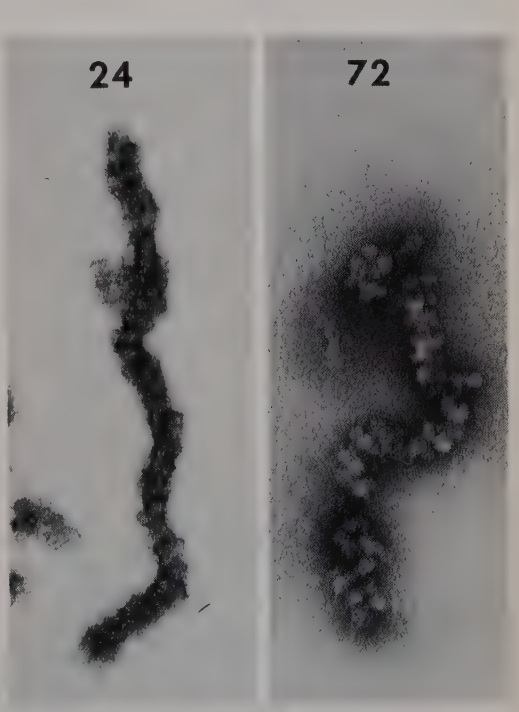

FIG. 3. Electron micrographs of large polysomes isolated from 24- and 72-hr embryonic chicken muscle cell cultures. Polysomes from the region of the gradient designated by the bracket in Fig. 2 were isolated and were stained with uranyl acetate as described in Materials and Methods. Polysomes from 24- and 72-hr muscle cell cultures appear similar in the electron micrograph and are similar to the myosin-synthesizing polysomes shown by Heywood *et al.* (1967). × 98,470.

(Fig. 4), and the sum of radioactivity in the slices making up this peak (indicated in Fig. 4) was used as the [^{3}H]leucine that had been incorporated into myosin heavy chains. Plotting this sum versus assay time (Fig. 5) showed that completion of myosin heavy chains increased very rapidly for the first 5 min, slowed between 5 and 7 min, and was finished by approximately 10 min. Because the assay contained no ribosomes other than those attached to the mRNA's of the polysomes and because the KCl concentration in the polysome isolation-buffer was high enough to extract muscle protein-specific initiation factors (Heywood, 1970), only chain-completion synthesis of myosin occurred in this system. To alleviate any possible differences in ability to initiate specific protein synthesis by ribosomes from 24- and 72-hr cultures, it was necessary that only chain-completion synthesis of myosin be occurring. The results shown in Fig. 5 are compatible with reports that about 4–5 min are required for movement of ribosomes along the entire length of a myosin messenger RNA (Coleman and Coleman, 1968; Herrmann *et al.*, 1970; Morris *et al.*, 1972).

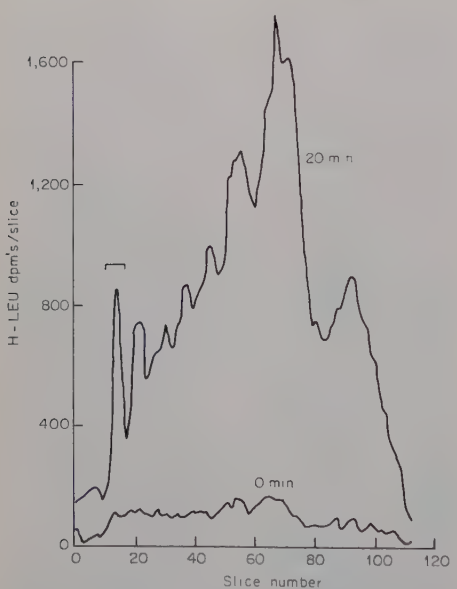

FIG. 4. Synthesis of myosin heavy chains by embryonic chicken leg muscle polysomes. Polysomes isolated from 14-day embryonic chicken leg muscle were incubated for 0 or for 20 min in the assay mixture described in Materials and Methods. The protein in these assay mixtures was dissolved in SDS and analyzed by SDS–polyacrylamide-gel electrophoresis. After staining, the gels were sliced and radioactivity in the slices was measured as described in Materials and Methods. The peak of radioactivity designated by the bracket migrated with a molecular weight of 200,000, and slices included under the bracket were used to calculate the 20-min time points in Fig. 5.

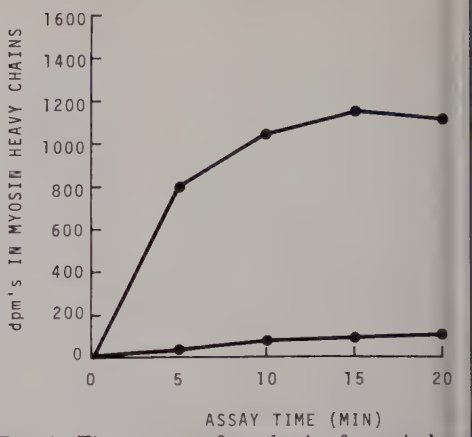

FIG. 5. Time course of synthesis of myosin hea chains by embryonic chicken muscle polysomes. P lysomes isolated from 14-day embryonic chicken l muscle were incubated in the cell-free assay m dium described in Materials and Methods. T lower curve is endogenous incorporation into myos that occurs when polysomes are omitted from th assay medium. Such a control was run with ea experiment, and this endogenous incorporation w subtracted from total myosin synthesis by pol somes from fused or from nonfused muscle cell cu tures.

When total polysomes isolated as described in Materials and Methods were subjected to sucrose gradient analysis after 2 min of assay time (i.e., after the accumulation of completed polypeptides had subsided), essentially all the ribosomes sedimented as 80S monomers or as subunits (Fig. 6), and no rapidly sedimenting polysomes remained. Hence, polysomes prepared according to the procedures used in this study were capable of actively moving along the messenger RNA's and completing the synthesis of polypeptides.

Because these preliminary experiment

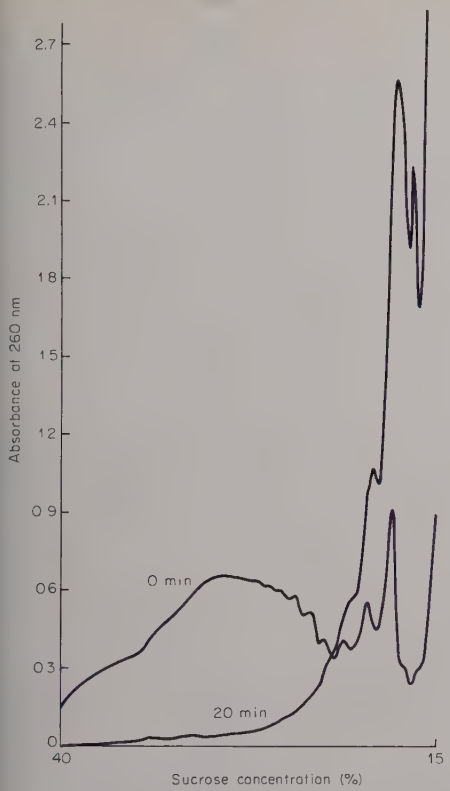

FIG. 6. Distribution of embryonic chicken muscle polysomes on a 15–40% sucrose gradient after these polysomes had been incubated for 0 or for 20 min in the assay mixture described in Materials and Methods. Gradients were centrifuged at 150,000g_{max} for 1.5 hr in a Beckman SW 41 rotor. Direction of sedimentation is from right to left.

estabished that polysomes prepared and assayed according to the procedures used in this study were able to complete the synthesis of peptide chains that had been initiated before their isolation, only 0 and 20-min time points were taken. The difference between the incorporation at 0 and 20 min was used as a measure of synthesis of myosin heavy chains. The lower curve in Fig. 5 represents endogenous incorporation into myosin heavy chains that occurred when polysomes were omitted from the assay mixture. Such a control was run with each experiment, and this control value was always subtracted from total myosin-synthesizing ability of the polysomes being analyzed.

The assay described in the preceding paragraphs was applied to total polysomes prepared from 24- and 72-hr muscle cell cultures to measure synthesis of myosin heavy chains by these polysomes. Because, as indicated in the preceding paragraph, the assay used in this study measures run-off of peptide chains that had been initiated before isolation of the polysomes, measurement of the amount of myosin heavy chain synthesis in these systems was a measure of amount of myosin polysomes in 24- and 72-hr cultures. Polysomes from both 24- and 72-hr cultures directed synthesis of polypeptides that migrated with a molecular weight of 200,000 on SDS–polyacrylamide-gel electrophoresis (Fig. 7). Furthermore, because the results in Fig. 7 are expressed on the basis of radioactivity incorporated into myosin heavy chains per 10^7 nuclei, the total polysomes from nonfused muscle cell cultures support synthesis of approximately half as much myosin as those from myotube cultures. It is possible that some of the myosin-synthesizing polysomes originally present in the cultured cells could have

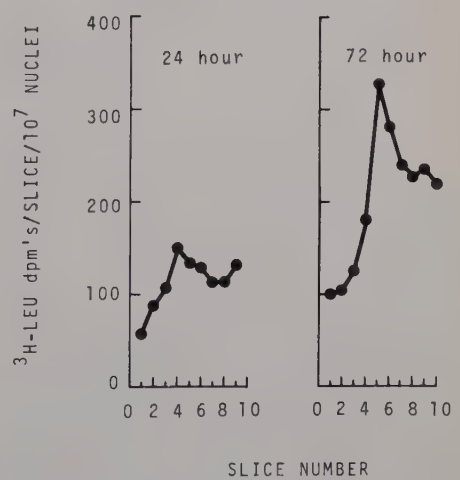

FIG. 7. Synthesis of myosin heavy chains by total polysomes isolated from 24- and 72-hr muscle cell cultures. Total polysomes were isolated, assayed, and the products analyzed by SDS–polyacrylamide-gel electrophoresis as described in Materials and Methods. The peak of [^{3}H]leucine incorporation supported by polysomes from either 24- or 72-hr cultures migrated exactly the same distance as myosin heavy chains.

remained in the pellet from the 12,000*g* centrifugation of the cell lysate in 0.5% Triton X-100 either because of incomplete cell lysis or because of physical entrapment. Any myosin-synthesizing polysomes remaining in these 12,000*g* pellets would not have been assayed and would therefore make the results shown in Fig. 7 inaccurate. To eliminate this possibility, the 12,000*g* pellets were reextracted in isolation buffer containing 0.5% Triton X-100 by mild homogenization with a Dounce homogenizer (A pestle). This homogenate was centrifuged at 12,000*g* for 10 min, and this second 12,000*g* supernatant fraction was also assayed for polysomes capable of synthesizing myosin heavy chains as described earlier. The results of these experiments suggest that, although a slightly larger percentage of myosin-synthesizing polysomes was pelleted at 12,000*g* in the myotube lysate (Table 1) than in the lysate from mononucleated myoblasts, this difference is small, and the data shown in Fig. 7 accurately indicate the relative quantities of myosin-synthesizing polysomes in 24- and 72-hr myogenic cell cultures. The summary of a number of experiments like the one in Fig. 7 indicates clearly that total polysomes from nonfused muscle cultures have about half the capability for incorporating [^{3}H]leucine into a 200,000-dalton polypeptide that polysomes from myotubes have (Table 2, left column).

Because a small but consistent level of fusion exists in 24-hr cultures (Fig. 1), it is critical to determine whether myosin-synthesizing polysomes in 24-hr cultures could originate entirely from the small number of fused myotubes present in those cultures. The number of nuclei within myotubes in the 24- and 72-hr cultures was measured by counting the nuclei in stained cultures as described in Material and Methods and scoring these nuclei as from either fused or nonfused cells. By using this measure of total nuclei present in multinucleated myotubes, and by using the total disintegrations per minute incorporated into myosin heavy chains by polysomes from 24- and 72-hr cultures (left column, Table 2), it is possible to calculate the amount of myosin polysomes that would be present per 10^7 nuclei in multinucleated myotubes if it were assumed that the mononucleated myoblasts in the 24- and 72-hr cultures contained no myosin

TABLE 1

MYOSIN SYNTHESIS BY POLYSOMES FROM 24- AND 72-HR MUSCLE CULTURES[a]

Source of polysomes	Percent of myosin-synthesizing polysomes in each supernatant[b]	
	24-Hr cultures	72-Hr cultures
First 12,000*g* supernatant fraction	94.9	84.9
Second 12,000*g* supernatant fraction	5.1	15.1

[a] Polysomes in the first 12,000*g* supernatant fraction from 24- and 72-hr muscle cultures were prepared and assayed as described in Materials and Methods. The pellet from the first 12,000*g* centrifugation was reextracted with isolation buffer plus 0.5% Triton X-100 to test for complete extraction of myosin-synthesizing polysomes. This second extract was also centrifuged at 12,000*g* for 10 min, and the supernatant fluid was assayed for polysomes capable of incorporating [^{3}H]leucine into myosin heavy chains as described in Materials and Methods.

[b] Percent of myosin-synthesizing polysomes = (myosin dpm by polysomes in each supernatant/sum of myosin dpm by polysomes in both supernatants) × 100.

TABLE 2

MYOSIN HEAVY CHAIN SYNTHESIS PER 10^7 NUCLEI AND PER 10^7 FUSED NUCLEI BY POLYSOMES FROM 24- AND 72-HR MUSCLE CELL CULTURES[a]

Culture age	[^{3}H]leucine in myosin heavy chains/10^7 nuclei (dpm)	[^{3}H]leucine in myosin heavy chains/10^7 fused nuclei (dpm)
24 Hr	498	12,475
72 Hr	914	1,406

[a] Incorporation of [^{3}H]leucine into myosin heavy chains and number of nuclei in single cells and in multinucleated myotubes were determined by the procedures described in Materials and Methods.

polysomes. The results of this calculation (right column, Table 2) show that the quantity of myosin polysomes per 10^7 fused nuclei would be nearly ninefold greater in myotubes in 24-hr cultures than in myotubes in 72-hr cultures if the small myotubes in 24-hr cultures were the only source of myosin-synthesizing polysomes. Because it seems incongruous to suggest that myotubes in 24-hr myogenic cell cultures should contain ninefold greater amounts of myosin polysomes than myotubes in 72-hr myogenic cultures, these results indicate that mononucleated muscle cells at 24 hr in culture contain significant levels of myosin polysomes.

The conclusion that mononucleated myoblasts contain approximately half as many myosin-synthesizing polysomes per 10^7 nuclei as multinucleated myotubes (Fig. 7 and Table 2) is partly based on the assumption that the average number of ribosomes per myosin-synthesizing polysome is identical in 24- and 72-hr muscle cell cultures, because total run-off of initiated peptide chains was used to measure amounts of myosin-synthesizing polysomes in the cells in these two cultures. To test this assumption, total polysomes from 24- and 72-hr cultures were fractionated on sucrose density gradients. The gradients were collected in eight different fractions, and the polysomes in each fraction were pelleted and then assayed for their ability to incorporate [^{3}H]leucine into myosin heavy chains as described in Materials and Methods (Figs. 8a and b). As expected (Heywood *et al.*, 1967), polysomes capable of synthesizing myosin were located near the bottom of the density gradient of total polysomes prepared from either 24- or 72-hr cells. Average size of the myosin-synthesizing polysomes from 24- and 72-hr myogenic cell cultures was similar, although the peak of myosin-synthesizing activity was in density gradient fraction 3 for polysomes from 1-day cultures but in density gradient fraction 2 for polysomes from 3-day cultures (Figs. 8a and b). These results indicate that mononucleated cells contain at least half as many myosin-synthesizing polysomes per 10^7 nuclei as multinucleated cells do. Indeed, if, as the data in Fig. 8 suggest, the average number of ribosomes per myosin-synthesizing polysome is actually slightly less in mononucleated cells

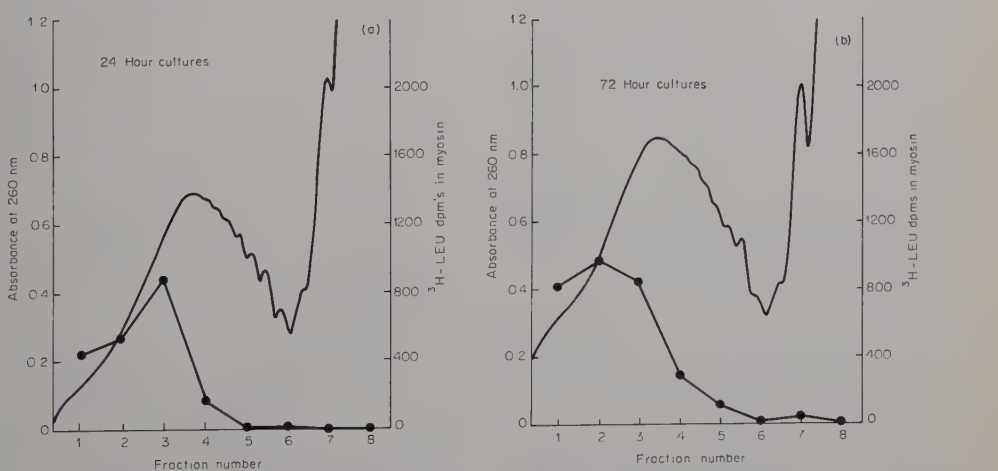

FIG. 8. Fractionation of total polysomes from 24-hr (a) and from 72-hr (b) muscle cell cultures on 15–40% sucrose gradients. Density gradients for the 24- and 72-hr fractions were centrifuged at the same time in identical tubes at 105,000g_{max} for 2.0 hr in a Beckman SW 25.2 rotor and were fractionated identically into eight different fractions each. Both 24- and 72-hr samples were obtained from cultures containing approximately 7×10^7 nuclei. Polysomes in each fraction were isolated and assayed for ability to synthesize myosin heavy chains as described in Materials and Methods. Direction of sedimentation is from right to left.

than it is in multinucleated cells, then our procedure for measuring myosin-synthesizing polysomes quantitatively by measuring run-off of initiated peptide chains would underestimate the number of myosin-synthesizing polysomes in mononucleated cells.

As indicated earlier, the 72-hr myogenic cell cultures contained approximately 1.5 times more total polysomes than the 24-hr cell cultures (left column, Table 3), and the results discussed in the preceding paragraph show that 72-hr myogenic cell cultures contain no more than two times more myosin-synthesizing polysomes than 24-hr myogenic cell cultures (left column, Table 2). These data can be used to estimate the change in proportion of total polysomes that is myosin-synthesizing polysomes which occurs between 24 and 72 hr in culture. The results of this calculation show that the proportion of myosin-synthesizing polysomes relative to total polysomes is only 1.2 times greater in 72-hr, multinucleated myogenic cell cultures than it is in 24-hr, mononucleated myogenic cell cultures (right column, Table 3). Yet, rate of myosin heavy chain synthesis is at least seven times higher in 72-hr myogenic cell cultures than in 24-hr cell cultures (Fig. 1). Consequently, cell fusion and the onset of bulk synthesis of myosin heavy chains is not accompanied by a dramatic increase in either the proportion of myosin-synthesizing polysomes relative to total polysomes or the total amount of myosin-synthesizing polysomes. Whether this ostensible disparity between rate of myosin heavy chain synthesis and amount of myosin-synthesizing polysomes indicates that myosin polysomes in multinucleated cells have a greater translation rate is presently unknown.

TABLE 3

TOTAL POLYSOME CONTENT AND MYOSIN HEAVY CHAIN SYNTHESIS PER UNIT OF POLYSOMES BY POLYSOMES FROM 24- AND 72-HR MUSCLE CELL CULTURES

Culture age	Total polysomes/10^7 nuclei (mg)	[^{3}H]leucine in myosin heavy chains/mg of total polysomes (dpm)
24 Hr	0.242	2,058
72 Hr	0.370	2,470

One factor which complicates interpretation of the data presented in this communication is that all muscle cultures are contaminated with fibroblasts which continue to proliferate for several days after extensive myoblast fusion has been completed. Because the proportion of fibroblasts increases somewhat between 24 and 72 hr in culture and because myosin synthesis and myosin-synthesizing polysomes are normalized on the basis of a constant number of nuclei, the values reported for 72-hr cultures are reduced to a greater extent than the values reported for 24-hr cultures. However, preliminary data obtained in this laboratory indicate that both the rate of myosin heavy chain synthesis and the amount of myosin-synthesizing polysomes in pure cultures of proliferating embryonic chicken skin fibroblasts may be similar to those values reported here for mononucleated myoblasts. If this is the case, then the values reported for rate of myosin synthesis and amount of myosin-synthesizing polysomes in mononucleated myoblasts are unaffected by fibroblast contamination, and these values in 72-hr cultures of multinucleated myotubes are not seriously affected. At any rate, the basic conclusion reached from the data presented in this communication that mononucleated myoblasts synthesize significant quantities of a polypeptide that has chemical properties identical to myosin is unaltered.

Another assumption on which the conclusion that multinucleated myotubes exhibit approximately seven times the rate of myosin heavy chain synthesis but only twice as many myosin-synthesizing polysomes as mononucleated myoblasts (Figs. 1, 7, Table 2) is based is that the only polypeptide that would survive precipitation in 0.025 *M* KCl followed by migration

with a polypeptide molecular weight of 200,000 on SDS–polyacrylamide-gel electrophoresis is myosin heavy chains. Although muscle contains several large proteins, most of them are easily resolved from myosin heavy chains on SDS–polyacrylamide gels and, in addition, these proteins have solubility properties unlike those of myosin. One possible exception may be the large subunit of nuclear RNA polymerase II (Weaver *et al.*, 1971).

The experiments described in the preceding paragraphs suggest not only that myosin messenger RNA is present in cultures of mononucleated myoblasts but that at least a portion of the myosin messenger RNA is associated with ribosomes to form polysomes approximately as large as the myosin-synthesizing polysomes from myotube cultures. These results are in general agreement with the data reported by two earlier groups of workers (Buckingham *et al.*, 1974; Yaffe and Dym, 1972). Yaffe and Dym (1972) found that myosin synthesis continues for several hours after fusion but then eventually decreases when cultures of dividing myoblasts are treated with actinomycin D. This result would be expected if the mononucleated myoblasts already contained myosin messenger RNA as we have found in the present study. Buckingham *et al.* (1974) demonstrated that a 26S, polyadenylic acid-containing RNA that hybridized to complementary DNA with kinetics identical to those of the myosin messenger RNA from which the complementary DNA was prepared could be isolated from fetal calf mononucleated myoblast cultures. Because our techniques would only detect myosin messenger RNA that was attached to ribosomes, we do not know whether our 24- and 72-hr cultures of embryonic chick myoblasts contained myosin messenger RNA unattached to ribosome in addition to the myosin polysomes that we found in these cells. In contrast to our results with embryonic chick myoblasts, however, Buckingham *et al.* (1974) did not report the presence of myosin polysomes in their fetal calf mononucleated myoblast cultures.

Several attempts by other investigators to isolate from mononucleated myoblasts a messenger RNA that would direct the cell-free synthesis of myosin heavy chains have been unsuccessful (Pryzbyla and Strohman, 1974); Pryzbyla *et al.*, 1973; Strohman *et al.*, 1974). In view of the recent accumulation of a large amount of evidence indicating that myofibrillar proteins are present in many different cell types, the isolation of myosin messenger RNA from mononucleated myoblasts is not surprising.

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Fine Structural Observations on the Origin and Associations of Primordial Germ Cells of the Mouse[1]

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This study explores the origin of primordial germ cells (PGCs) of the mouse and examines their morphology and associations with other cells during early development. PGCs have been selectively stained by the alkaline phosphatase histochemical reaction and viewed by light and electron microscopy from the time they are first detectable in the yolk sac endoderm until they enter the gonadal ridges. There are conflicting reports as to whether the PGCs originate from endodermal cells or whether they originate elsewhere and subsequently enter the endoderm. The observations in the present study favor the premise that PGCs of the mouse do not originate in the endoderm. Furthermore, it was observed that PGCs undergo specific changes in morphology during the developmental period studied and this was interpreted to mean that, although PGCs are set aside early in development as a distinct cell line, they also continue to become more specialized within time. The germ cell line is rather unusual in that it does not exist as a discrete tissue but, instead, resides within various other tissues during its life history. This apparent dependence upon somatic cells is maintained even in adult animals and may be important in serving to maintain or modify the environment of the germ cells.

INTRODUCTION

Much of the early life history of the germ cell line in mammals remains to be elucidated. There once was considerable debate as to whether the germ cells arose within the gonad or whether they originated in an extragonadal site (reviewed by Heys, 1931; Everett, 1945; Nieuwkoop, 1949). It has since been shown by experimental (Everett, 1943; Mintz and Russell, 1957) and histochemical studies (McKay *et al.*, 1953; Chiquoine, 1954) that the germ cell line begins with the primordial germ cells (PGCs) first observed in the yolk sac endoderm. These cells are incorporated into the hindgut and subsequently migrate through the dorsal mesentery to the gonadal ridges. Thus, the germ cell line is continuous from early embryonic through adult stages of life (reviewed by Brambell, 1956; Franchi *et al.*, 1962). However, just where and when the PGCs originate remains to be determined.

There are conflicting reports as to whether the PGCs originate from endodermal cells or whether they originate elsewhere and subsequently enter the endoderm. Chiquoine (1954) was first able to identify PGCs of the mouse with the alkaline phosphatase histochemical technique when they lay at the caudal end of the primitive streak, in the root of the allantoic mesoderm and within the yolk sac splanchnopleure. Since most of the germ cells were seen in the yolk sac, he proposed that PGCs had their origin in the endodermal cell layer. From similar observations, Mintz and Russell (1957) also concluded that PGCs of the mouse originate in the endoderm. However, Ozdzenski (1967) used the same techniques but reported that PGCs of the mouse were first detected within the embryonic rudiment of the allantois. PGCs were present only later in the yolk sac endoderm underlying the primitive streak, suggesting to Ozdzenski that PGCs had a mesodermal origin. Also, Spiegelman and Bennett (1973) noted that

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PGCs in the hindgut endoderm of mouse embryos resembled adjacent splanchnic mesodermal cells and suggested that some PGCs appeared to be migrating in from the mesoderm.

Three reports have appeared recently which discuss the fine structural appearance and associations of PGCs in mouse embryos. Jeon and Kennedy (1973) used the alkaline phosphatase technique to identify PGCs and studied them from their detection in the wall of the hindgut until they reached the gonadal ridges. In the other studies, PGCs were identified either due to their affinity for toluidine blue as seen by light microscopy or due to their cytoplasmic density as determined by electron microscopy (Spiegelman and Bennett, 1973; Zamboni and Merchant, 1973). However, none of these studies examined PGCs by electron microscopy at the important early stage of embryonic development when they first appear in the extraembryonic endoderm of the yolk sac.

The purposes of the present study were to explore further the origin of PGCs and to examine their morphology and associations with other cells during the early part of embryogenesis. To do this, the PGCs of mouse embryos were selectively stained by the alkaline phosphatase histochemical reaction and viewed by light and electron microscopy. This allowed PGCs to be studied at the electron microscopic level during the earliest period of development in which they have been detected previously by light microscopy. In addition, the ultrastructural features of PGCs and associated somatic cells were examined from the time of earliest demonstration of PGCs until the time they settled in the gonadal ridges. The purpose of this was to determine whether there were observable changes in the PGCs during this interval and whether there were morphological indications of specific interactions between germ cells and somatic cells. Finally, attempts were made to gain new information about PGCs by examining them in squashed tissue preparations after they had been fixed and reacted for alkaline phosphatase enzymatic activity.

MATERIALS AND METHODS

White Swiss mice (Simonsen Labs, Gilroy, Calif.) were used in this study. Females were placed in cages with males overnight and examined for vaginal plugs the next morning, with the day on which a plug was found being considered day 1 of gestation. The gestational ages given in this study are approximate, however, because the time of fertilization is accurate only to within 12 hr and because it was observed that there is appreciable variation in stages of development within a litter. Consequently, developmental features such as numbers of somites, degree of neural tube closure and gut formation were used to stage individual embryos. In order to remove the embryos, pregnant females were anesthetized with ether and the uterine horns transferred to a dish containing mammalian Ringer's solution. Day-8 embryos were processed intact within their accompanying extraembryonic membranes, but older embryos were separated from their extraembryonic membranes and the upper body trimmed away before subsequent processing.

Fixation was carried out at room temperature by placing the embryos in 3 ml of 300 mos*M* cacodylate buffer (Sigma Chemical Co.) adjusted to pH 7.4, to which was added dropwise 2 ml of 6.25% glutaraldehyde (Electron Microscopy Sciences) in water (Morel *et al.*, 1971). This resulted in a final buffer of 180 mos*M* and a final glutaraldehyde concentration of 2.5% (Bone and Denton, 1971). After 20 min, the embryos were rinsed for 1 hr in 180 mos*M* cacodylate buffer to remove all traces of the fixative. In some cases the tissues were then embedded in 7% agar and sections 100 μm in thickness were cut with a Smith–Farquhar tissue chopper (Sorvall Co.). These sections or intact embryos were then placed in an alkaline phosphatase

reaction solution at pH 8.2, containing sodium β-glycerophosphate (Sigma Chemical Co.) and lead nitrate (K and K Laboratories, Inc.) in Tris–maleate buffer (Sigma Chemical Co.), prepared according to the method of Hugon and Borgers (1966). The tissue was incubated for 45 min–125 hr at 37°C, the longer time being necessary for younger embryos. Tissues to be examined by electron microscopy were rinsed in 180 mos*M* cacodylate buffer and placed in a solution of three parts of 300 mos*M* cacodylate buffer and two parts of 2% osmium tetroxide for 2 hr at room temperature. They were then washed in distilled water, dehydrated through a series of ethyl alcohols, transferred to propylene oxide and embedded in Epon epoxy resin (Luft, 1961). To prepare light micrographs, sections 1 μm thick were stained with 0.1% basic toluidine blue and photographed using an orange filter (Wrattan 22). For electron microscopy, thin sections were stained with uranyl acetate and lead citrate (Reynolds, 1963) and examined in a Philips EM 200.

Portions of embryos from 8 to 13 days gestation were fixed and reacted for alkaline phosphatase according to the methods described above, but then in addition were immersed in a 1% solution of ammonium sulfide for 2 min in order to enhance the visibility of the reaction product for light microscopy. They were then rinsed in 180 mos*M* cacodylate buffer, placed on a slide in a drop of buffer and gently flattened with a coverslip. Some tissues were squashed first, then fixed and reacted beneath the coverslip. These preparations were observed and photographed with a Zeiss Universal microscope equipped with Nomarski differential interference contrast optics.

OBSERVATIONS

Morphology of PGCs. In later stages of development (after day 9 of gestation), PGCs are readily identifiable in 1-μm sections viewed by light microscopy because they are large cells with a distinct affinity for basic toluidine blue stain (Figs. 7 and 11). There are also a number of characteristics which allow PGCs to be recognized at the electron microscopic level. Prior to their location of PGCs in the gonad, their nuclei are irregular or horseshoe shaped and contain prominent nucleoli. The cytoplasm contains sparse endoplasmic reticulum, numerous free ribosomes and large, rounded mitochondria with few cristae (Figs. 9 and 12). Also, the cytoplasm of PGCs has a higher affinity for the electron-dense stains used than does that of surrounding cells, possibly due to differences in the make-up of the ground cytoplasm and the high concentration of ribosomes present in PGCs.

In addition to these general characteristics, PGCs may be identified more specifically by the presence of distinct, dense-cored vesicles in their cytoplasm. These round, membrane-bounded vesicles contain an electron-dense core which is surrounded by a finely flocculent zone. The peripheral area sometimes appears to be empty, suggesting that the dense core may be the precipitated remnant of material formerly filling the vesicle or that material in the periphery has been removed during processing. These vesicles vary from 0.2 to 0.35 μm in diameter and serve as reliable, readily identifiable markers for the detection of PGCs (Figs. 8, 12 and 15).

Although the features described above allow the typical PGC to be readily identified, it became apparent early in this study that not all cells could be classified unequivocally as somatic or germinal on these characteristics alone. This was particularly true for cells in the yolk sac endoderm. Other workers had previously used the alkaline phosphatase histochemical reaction at the light microscopic level to identify PGCs in early stages of embryonic development. When this was applied at the light and electron microscopic level in this study, it was found that PGCs could

be identified with either the light microscope (Figs. 7 and 11) or the electron microscope (Figs. 4, 8 and 13) by the rim of lead phosphate reaction product deposited at the periphery of the cell. In addition, PGCs could be observed directly in whole mounts of embryonic tissue when the alkaline phosphatase reaction was amplified by subsequently complexing it with ammonium sulfide. The alkaline phosphatase reaction appears to be a preferential label for PGCs, allowing them to be distinguished easily from surrounding cells. Although the cells of the neural tube react as well, it is a much weaker reaction and occurs well away from the areas which include PGCs.

Days 8–8.5 of gestation. Embryos varying from the presomite to the 4-somite stages of development were fixed during the 8–8.5-day period of gestation. This is the earliest embryonic stage at which PGCs were identified. In the youngest embryos, one can identify the headfold and the amnion as well as the allantois, which is just beginning to form from the posterior end of the primitive streak (Fig. 1). There is no hindgut, but there may be a slight indentation in the yolk sac endoderm which is presumably a first indication of hindgut formation. PGCs are detectable by light microscopy because they have a faint accumulation of the alkaline phosphatase reaction product at their periphery and they are slightly more basophilic than the surrounding endodermal cells (Fig. 2). The alkaline phosphatase reaction product is also the major identifying characteristic of PGCs at the electron microscopic level, where it is seen as a discontinuous accumulation of small electron-dense particles at the plasma membrane (Figs. 3–5). In some PGCs the reaction product is present in the Golgi apparatus as well (Fig. 3). Using these means, PGCs may be detected in the caudal portion of presomitic embryos, lying within the endoderm of the yolk sac, in the area destined to be incorporated into the hindgut (Fig. 2). Although some PGCs are present in the allantois in later stage embryos, in presomitic embryos they have been observed by electron microscopy only in the endoderm. It is estimated from electron micrographs that the PGCs are less than 12 μm in diameter at this stage.

It is fortunate that the alkaline phosphatase reaction provides a good method for the identification of PGCs at this stage, for other features that are characteristic of PGCs at later stages of development are less reliable. For example, it has been observed that small vesicles containing a suggestion of a dense core are present in PGCs in presomitic embryos but similar vesicles

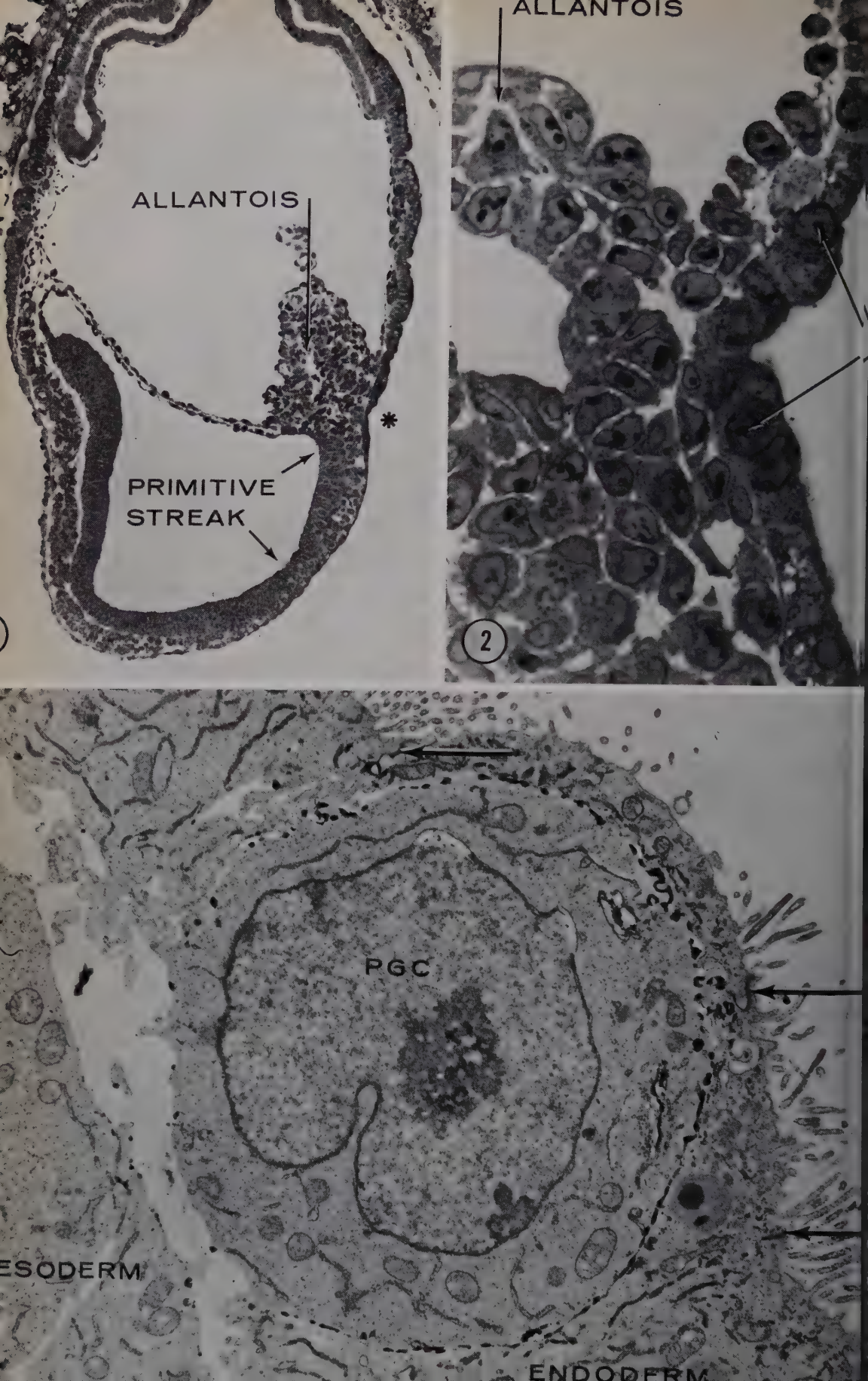
FIG. 1. Sagittal section of a day-8 mouse embryo. The headfold region is present to the left and the allantois may be observed at the caudal end of the primitive streak to the right. The PGCs are found within the yolk endoderm, in or near the indentation (asterisk) adjacent to the allantois and primitive streak. The indentation appears to be the first indication of hindgut formation. × 110.

FIG. 2. A higher-magnification light micrograph of the indented area in another embryo at a stage of development similar to that shown in Fig. 1. The PGCs are identifiable within the endoderm of this area because of their basophilia and the presence of a thin rim of alkaline phosphatase reaction product deposited at their periphery. × 740.

FIG. 3. Electron micrograph showing a PGC within the endoderm of a day-8 embryo. The yolk cavity is to the right, mesoderm to the left. Alkaline phosphatase reaction product is present on the surface of the PGC, although only in trace amounts on the portion toward the mesoderm. Reaction product is also present in some of the cisternae of the Golgi apparatus. The endodermal cells are attached to each other by junctions (arrows) at their lateral margins adjacent to the yolk sac lumen. The endodermal cells have small accumulations of alkaline phosphatase reaction product on their microvilli. × 9300.

FIG. 4. A PGC in the yolk sac endoderm of a day-8 embryo has a small pseudopodium (arrow) extending into the space between endoderm and mesoderm. The endoderm lacks a basal lamina. The cells of the endoderm contain far more endoplasmic reticulum, vesicles and mitochondria than the PGC. × 7750.

FIG. 5. Another PGC of a day-8 embryo is located between the yolk sac endoderm and the mesoderm. The endodermal cells have junctions at their apical margins and large intracellular spaces more basally. × 8700.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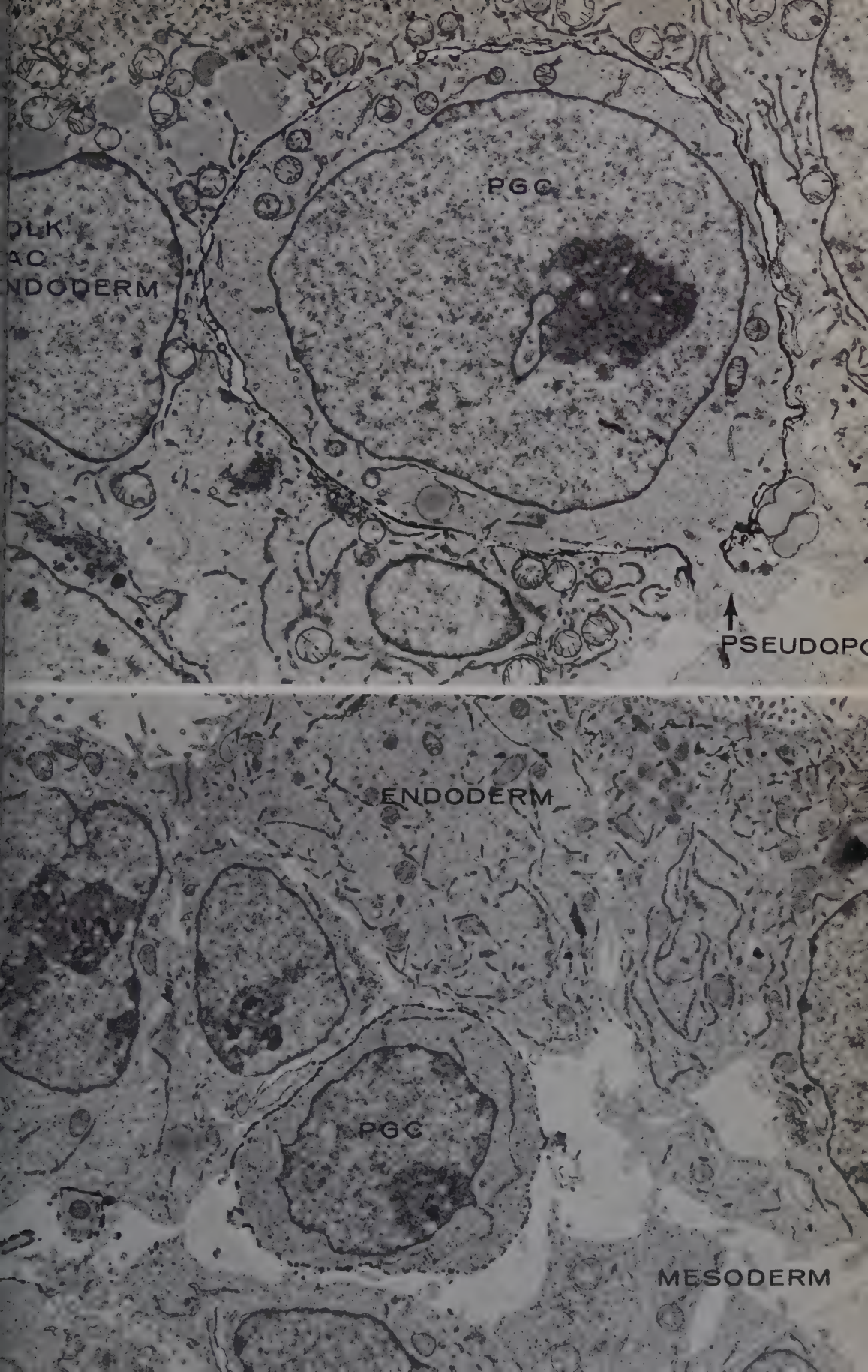
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also appear to be present in surrounding cells which lack the reaction product. Also, the mitochondria of PGCs are not different from those found in adjacent cells at this stage. Furthermore, although the nuclei of PGCs are often horseshoe shaped or indented, visualization of this characteristic is dependent upon the plane of section. It would not seem from these observations that there are any other specific morphological characteristics of the nucleus or cytoplasm that are more useful for the identification of individual PGCs at this period of development than the alkaline phosphatase reaction product.

Once the PGCs are identified, it becomes more apparent that they differ in several ways from the endodermal cells of the visceral yolk sac with which they are associated. The endodermal cells are cuboidal or columnar shaped while the PGCs are usually round. Also, only the endodermal cells are connected to each other by an apparently continuous junctional band at the lateral margin of their apical end, excluding the PGCs from contact with the yolk cavity (Fig. 5). The apical surface is microvillous and the cytoplasm just deep to the microvilli contains a variety of vesicles, suggestive of pinocytotic activity (Figs. 3–5). It should be noted that the microvilli of the endodermal cells may show an accumulation of alkaline phosphatase reaction product, although it is less dense than that on the surface of the PGCs (Figs. 3 and 4). The endodermal cells also contain a greater amount of endoplasmic reticulum and more lipid droplets and mitochondria than the PGCs. The endodermal cells change quite noticeably in appearance between the portion applied to the embryonic disc and the portion that lines the remainder of the yolk sac. Those cells that underlie the embryonic disc form a low epithelium and have fewer and shorter microvilli, appearing less active than those in the extraembryonic region (Figs. 1 and 2). The PGCs usually lie within the portion of the yolk sac where these two types of cells converge at the caudal end of the embryonic disc.

In contrast to the endodermal cells, the mesodermal cells underlying the yolk sac endoderm are rather similar to PGCs in morphology. Although the mesodermal cells stained less densely than PGCs, they have a sparse amount of endoplasmic reticulum, modest numbers of mitochondria and irregularly shaped nuclei, like PGCs (Figs. 3 and 5). These similarities are of interest because the endoderm lacks a basal lamina at this stage, indicating that there is no structural barrier preventing PGC movement either into or out of the endoderm, and sometimes PGCs do lie outside of the endoderm (Fig. 5) or extend processes out of the endoderm toward the mesodermal cells (Fig. 4). Frequently, the portion of a PGC extending out of the endoderm lacks an accumulation of reaction product (Figs. 3 and 4).

Days 9–9.5 of gestation. The hindgut has closed and become separated from the yolk sac in most embryos examined at 9–9.5 days of gestation (Fig. 6). The midgut region is open to the yolk sac cavity and is continuous laterally with the yolk sac splanchnopleure. The neural groove has not yet begun to close in the area of the hindgut (Fig. 6). By this time PGCs have most of the same morphological features that are seen in later stage PGCs. In particular, the dense-cored vesicles are now a prominent characteristic of PGCs and often lie in the vicinity of the Golgi apparatus (Figs. 8 and 9). However, PGCs are most easily detectable by light and electron microscopy in these embryos when they have the alkaline phosphatase reaction product on their surface (Figs. 7–9). A majority of PGCs at 9–9.5 days are distributed throughout the epithelium of the hindgut (Fig. 7), with a few being detected in the adjoining allantois or in the splanchnopleure of the midgut.

The cells of the hindgut epithelium are columnar and have microvilli on their luminal surface (Figs. 8 and 9). They are

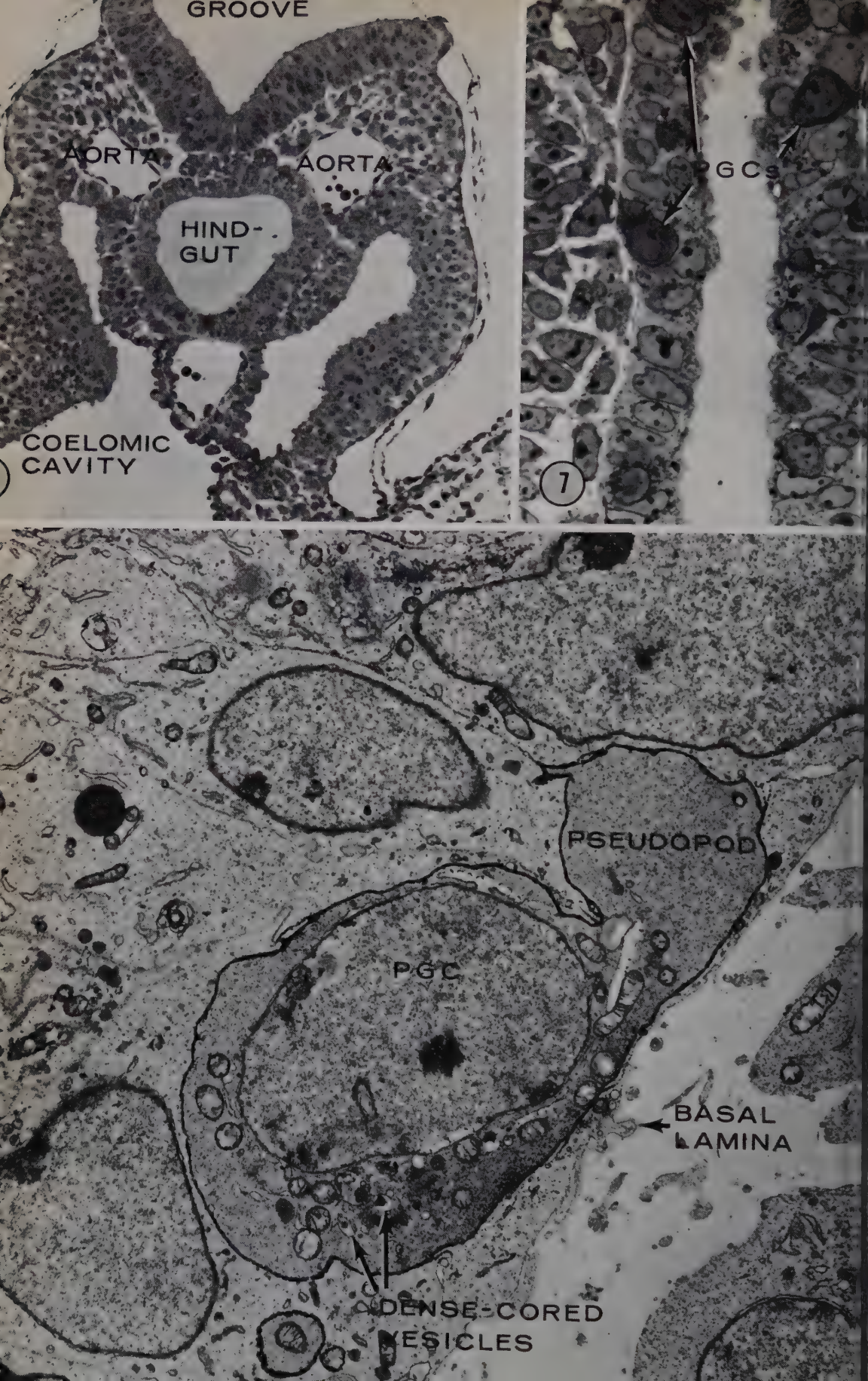
usually polarized, with the nucleus situated toward the basal end of the cell and elements of granular endoplasmic reticulum and lipid droplets lying in the cytoplasm at the luminal end. The epithelium is underlain by a complete basal lamina and there are extensive junctions at the lateral margins of the cells just below their luminal surface. Confined between the adluminal junctions and the basal lamina, the PGCs distort and displace the cells of the hindgut epithelium. A number of PGCs have been observed within the epithelium with large, blunt pseudopodia that are devoid of organelles, the organelles being clustered near the nucleus (Fig. 8). These PGCs, which have the characteristics of migrating cells, are most frequently observed in sections cut in a cranial to caudal embryonic plane and appear to be migrating cranially within the epithelium.

Days 10–11 of gestation. During days 10–11 of gestation the gut forms a closed tube attached to the dorsal body wall of the embryo by the mesentery. Gonadal ridges are beginning to develop just medial to the mesonephros as small bulges on the dorsal coelomic wall (Fig. 10). PGCs may be identified by the same morphological characteristics and alkaline phosphatase distribution pattern as the PGCs described before (Figs. 11–13). At the early part of day 10, PGCs are beginning to leave the epithelium of the hindgut and to move through the dorsal mesentery to the developing gonadal ridges (Fig. 11). By day 11 most PGCs are within the dorsal mesentery. It may be seen, even by light microscopy, that PGCs are elongate cells with a broad, blunted pseudopodium which is usually directed dorsally (Fig. 11). On electron micrographs PGCs commonly have an irregular outline with a pseudopodium at one end and an indented nucleus at the other end. Most cytoplasmic organelles are clustered at one side of the nucleus (Fig. 12).

Once they have left the epithelium of the hindgut and before they settle in the gonadal ridges, PGCs do not appear to associate preferentially with a particular cell type in the dorsal mesentery. During day 10, PGCs are seen in the mesenchyme (Fig. 13) between the aorta and the coelomic epithelium migrating along the base of the coelomic epithelium (Fig. 12). A day later, when larger intercellular spaces are apparent, PGCs also move freely through the loose mesenchyme.

The cuboidal cells of the coelomic epithelium lack microvilli but the organelles and nuclei are distributed similarly to those of the gut epithelium. There are generous amounts of mitochondria and endoplasmic reticulum associated with the apical cytoplasm, again suggesting possible absorptive or synthetic activities (Fig. 12). Although the coelomic epithelial cells lack a continuous basal lamina at this time, they are connected to each other at the lateral margins of their apical surface by an apparently continuous junctional band (Fig. 12). PGCs may displace the basal portion of these cells but probably are prevented from entering the coelom by the junctions. PGCs may also indent or distort the mesenchymal cells which they contact during this same period.

Days 12–13 of gestation. PGCs become located within the gonadal ridges on days 12 and 13 of gestation (Fig. 14); the ridges are seen as elongate bulges of the coelomic epithelium on the dorsal body wall, lying lateral to the aorta, medial to the mesonephros and inferior to the liver anlagen. PGCs observed to be entering the gonadal ridges usually show the characteristics of migratory cells. However, PGCs in the closely packed tissue just dorsal to the coelomic epithelium overlying the gonadal ridge lack pseudopodia and display a random distribution of organelles and a round, central nucleus. Estimates based on preliminary measurements of electron micrographs indicate that PGCs are approximately 14 μm in diameter at this stage. Although the gonads are sexually undifferentiated, the PGCs are closely as-


GROOVE
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VESICLES

sociated with somatic elements which will presumably become either Sertoli or follicular cells.

At this stage, some PGCs do not show a strong affinity for toluidine blue by light microscopy and some are less dense than earlier-stage PGCs observed by electron microscopy. An additional feature for identification of PGCs at this stage is the lack of a rim of heterochromatin adjacent to the nuclear envelope, as is seen in somatic cells (Fig. 14). It is common to observe groups of PGCs at this time (Fig. 15) which appear to be attached to each other by focal junctions. Focal junctions are also present between PGCs and the adjacent somatic cells.

PGCs in whole mounts. The region containing PGCs was dissected from previously fixed day-8–13 embryos, subjected to the alkaline phosphatase reaction and ammonium sulfide treatment, compressed between a coverslip and slide and viewed with differential interference contrast optics. In the youngest embryos studied, having four or fewer somites, it was difficult to distinguish precisely where the PGCs were located due to the weakness of the reaction and the lack of well-defined tissues. However, it could be observed that most of the PGCs are clustered in an area below the base of the allantois, apparently in the portion of the endoderm which is just beginning to invaginate. Also, a few PGCs are found in the allantois or in the splanchnopleure of the yolk sac lateral to that being incorporated into the gut. There are relatively few PGCs present and they are scattered in the endoderm; from 20 to 100 PGCs per embryo were identified due to their accumulation of the enzymatic reaction product in the 12 embryos studied. These numbers are comparable to the numbers of PGCs found in serially sectioned embryos at the same stage (Mintz and Russell, 1957).

PGCs react strongly for alkaline phosphatase and are readily observed in tissues from embryos fixed in later stages of development. On day 9 of gestation, PGCs are found distributed throughout the entire length of the hindgut and in the closing midgut (Fig. 16) and a few are observed in the dorsal mesentery. They are irregular in shape and some have thin processes extending between somatic cells. From 170 to 350 PGCs per embryo were found in the ten embryos examined at this stage. These numbers also agree with the numbers of PGCs found in the gut of sectioned mouse embryos of similar developmental stage (Mintz and Russell, 1957; Bennett, 1956). By days 10 and 11, most PGCs have moved into the dorsal mesentery. They appear to have blunted pseudopodia or cytoplasmic processes extending between surrounding cells and frequently are observed associated in pairs throughout the tissue. By this time, PGCs are too numerous to accurately count. On days 12 and 13 PGCs are rounded and lay within the gonadal ridges, usually being aggregated into rouleaux of two to eight or more cells just deep to the coelomic epithelium (Fig. 17).

DISCUSSION

Characteristics of PGCs. Many of the fine structural characteristics of PGCs ob-

FIG. 6. Light micrograph of a transverse section of a day-9 embryo demonstrating the relationship of the hindgut to the neural groove, coelomic cavity and aorta. × 160.

FIG. 7. In a longitudinal section through the hindgut of a day-9 embryo, the PGCs are identifiable by their basophilia and the reaction product at their surface. Some PGCs appear rounded and others are elongate in this light micrograph. × 640.

FIG. 8. By electron microscopy, a PGC in the hindgut of a day-9 embryo is seen to have more densely staining cytoplasm than the surrounding gut epithelial cells. The lumen of the hindgut is at the upper left, and the basal lamina separating epithelium from connective tissue is at the lower right. The PGC has a blunt pseudopodium devoid of membranous organelles, demonstrating the characteristics of a migratory cell. Dense-cored vesicles are visible in the vicinity of the Golgi apparatus. × 5100.

served in this study are similar to those reported recently in other studies on PGCs of the mouse (Jeon and Kennedy, 1973; Spiegelman and Bennett, 1973; Zamboni and Merchant, 1973) and of the rat (Eddy, 1974; Eddy and Clark, 1975). However, in this study, PGCs have been examined at an earlier stage of development than in any previous fine structural study. It has been found, by following the PGCs from the time they are first detectable with the alkaline phosphatase technique in the yolk sac endoderm until they enter the gonadal ridge, that there are significant changes in the PGCs during the early life history of the germ cell line in mice.

It was noted in this study that PGCs in the yolk sac endoderm average less than 12 μm in diameter and are about 14 μm in diameter when they reach the gonadal ridges. PGCs also show changes in shape and in distribution of organelles during this period, probably due to changes in degree of migratory activity (discussed below). In addition, the cytoplasmic organelles seem to increase in number, the Golgi apparatus becomes more prominent and dense-cored vesicles appear and become characteristic features of PGCs in the later stages of development. Finally, the reaction product of alkaline phosphatase changes from a scattered distribution on early PGCs to an even deposit on later-stage PGCs reacted for the same amount of time. These observations are interpreted to mean that although the PGCs are set aside early in development as a distinct cell line, they also continue to become more specialized with time.

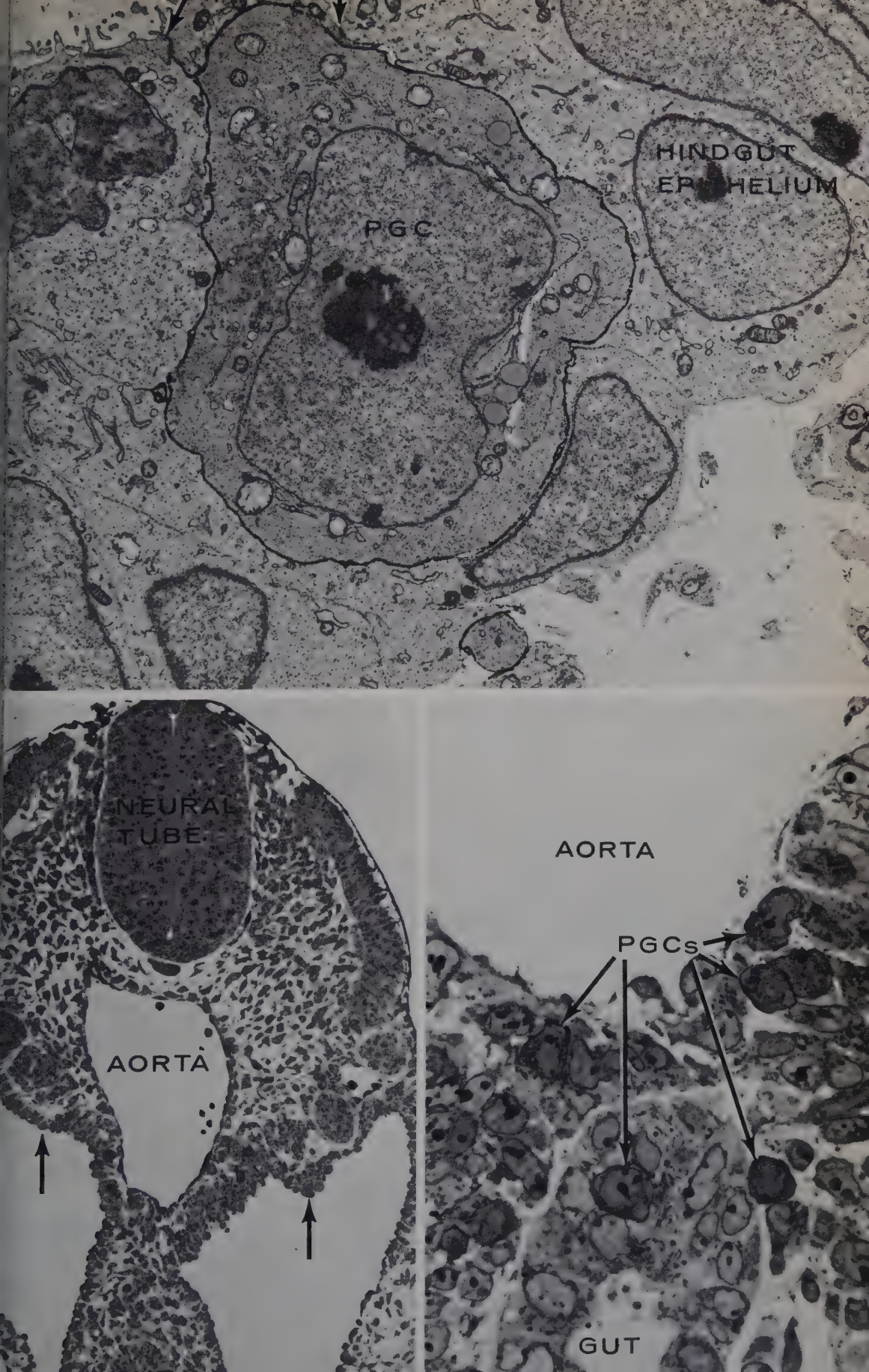
It would be useful if there were distinct fine structural features available for the unambiguous identification of PGCs at an early stage of development. In the early phases of this study it seemed possible that the dense-cored vesicles might serve this role. They were apparently confined to members of the germ cell lines were easily recognizable and were relatively numerous. Furthermore, they had been observed previously in other studies on PGCs of the mouse (Zamboni and Merchant, 1973; Jeon and Kennedy, 1973; Spiegelman and Bennett, 1973), PGCs of the rat (Eddy, 1974; Eddy and Clark, 1975), oogonia and oocytes of the mouse (Odor and Blandau, 1969) and spermatogonia and oogonia of the rat (Eddy, 1974). However, this study showed that the dense-cored vesicles are a suitable characteristic for identifying PGCs only from the time the PGCs are present in the hindgut until after they settle in the gonadal ridges. Until something is known about the formation and nature of the dense-cored vesicles, it is possible only to speculate that they may play a role in the unique function of the germ cell line.

The alkaline phosphatase histochemical reaction was used in this study because it appeared to provide the best morphological means for the identification of PGCs. Earlier workers suggested that the reaction product was distributed throughout the cytoplasm (Chiquoine, 1954; Mintz, 1959), but it is apparent from this and other studies (Jeon and Kennedy, 1973; Eddy and Clark, 1975) that the enzyme is located at the plasma membrane of PGCs. Reaction product also was noted in the Golgi apparatus of PGCs in the yolk sac endoderm in

FIG. 9. In a day-9 embryo, PGCs may extend nearly the full height of the epithelium, as seen here, or may be situated near the base of the epithelium (Fig. 8). However, they are confined within the epithelium, apparently by the adluminal junctions and the basal lamina. × 6100.

FIG. 10. This transverse section of a day-10 embryo passes through the developing gonadal ridges on the dorsal body wall (arrows). The PGCs are beginning to migrate from the gut, at the lower edge of this light micrograph, through the dorsal mesentary to reach the gonadal ridges. × 170.

FIG. 11. In a light micrograph of higher magnification than Fig. 10, the PGCs may be recognized within the epithelium of the gut and the adjacent connective tissue of a day-10 embryo due to their densely stained cytoplasm and surrounding reaction product. × 700.


HINDGUT
EPITHELIUM
PGC
NEURAL
TUBE
AORTA
AORTA
PGCs
GUT

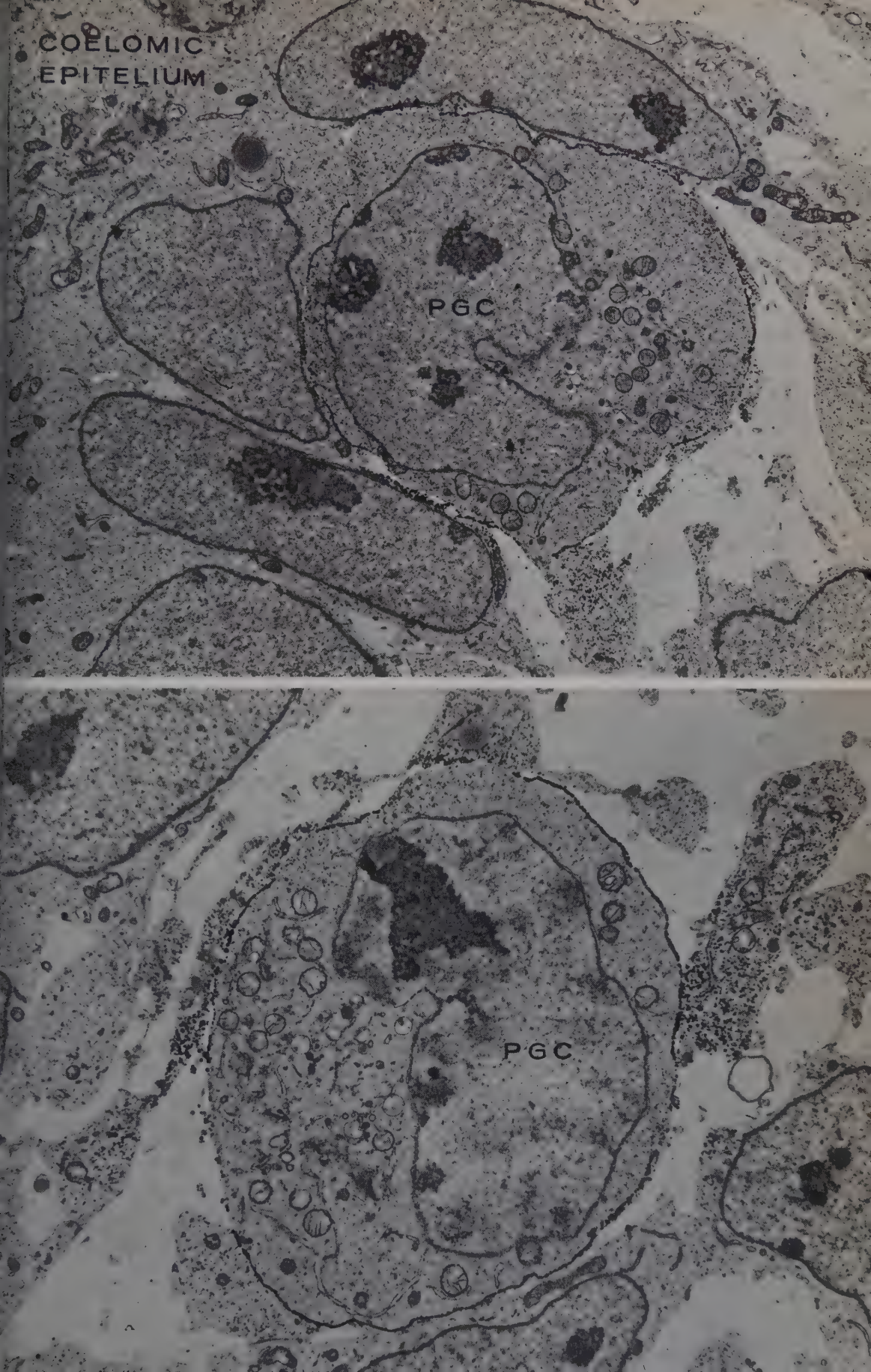
this study and of PGCs in the gonadal ridge in the study by Jeon and Kennedy (1973). It has been suggested that the Golgi apparatus packages the enzyme for transport to the plasma membrane (Jeon and Kennedy, 1973). Since the alkaline phosphatase enzyme molecule has a substantial carbohydrate component (Fishman and Ghosh, 1968), it may well be that this portion is added to the molecule during its transit through the Golgi apparatus (Neutra and Leblond, 1966). However, it is not known what role the alkaline phosphatase serves in PGC function and additional work will be needed before the enzyme can be linked to some specific activity of these cells.

One of the more prominent characteristics of PGCs is their migratory ability. Taking special note of certain cellular features, such as the polarized cytoplasm, the presence of blunt pseudopodia and the variety of routes taken by PGCs, Witschi (1948) concluded that PGCs in human embryos move individually through the tissues and into the gonadal ridges. It was subsequently demonstrated in studies on living PGCs that these cells were capable of active amoeboid motion (Sapsford, 1962; Blandau *et al.*, 1963). Although previous fine structural studies have noted that PGCs show the characteristics of migrating cells (Spiegelman and Bennett, 1973; Zamboni and Merchant, 1973; Eddy and Clark, 1975), the present study indicates that the migratory ability changes with development. For example, the morphology of PGCs in the yolk sac endoderm suggests that they are sedentary cells for the most part. Thus, PGCs are probably carried into the hindgut by the invagination of the surrounding endoderm rather than by independent migration. Also, the nonmigrating PGCs in the gonadal ridge are similarly round and have a homogeneous distribution of organelles. On the other hand, PGCs in the hindgut and in the dorsal mesentery frequently have the morphology of migratory cells. Although Jeon and Kennedy (1973) concluded that amoeboid motion alone cannot account for the movement of PGCs through the mesentery, sampling limitations of electron microscopic studies, the effects of fixation and the plane of the section examined could influence the interpretation. In this study, PGCs observed in the hindgut epithelium cut along the cranial–caudal axis exhibited migratory features more frequently than PGCs in sections transverse to that axis. This confirms the suggestion that the detection of migratory features in these cells require optimal sections (Spiegelman and Bennett, 1973).

Origin of PGCs. The origin of PGCs in mammals continues to be an unresolved issue because of conflicting observations and the paucity of experimental evidence. The two proposals that have been made are that PGCs may arise in the yolk sac endoderm (Chiquoine, 1954; Mintz and Russell, 1957) or that they may originate in the mesoderm and become located secondarily in the endoderm (Ozdzenski, 1967; Spiegelman and Bennett, 1973). In the present study, the earliest time that PGCs were detected by means of the alkaline phosphatase reaction was when they were present in the endoderm of the yolk sac in the presomite period of develop-

FIG. 12. Electron micrograph of a PGC associated with the coelomic epithelium of a day-11 embryo. The PGC appears to be displacing some of the epithelial cells with its leading pseudopodium and the mitochondria and dense-cored vesicles are clustered near the indented nucleus. The coelomic epithelium lacks a basal lamina, but the cells are attached by adluminal junctions. × 5600.

FIG. 13. This PGC in a day-11 embryo appears to be moving through the connective tissue, in contact with several cells at various points on its surface. The leading edge appears to be towards the upper right with that part of the cytoplasm having relatively few membranous organelles and the opposite cytoplasm containing an aggregation of organelles. Here, as in Fig. 12, an indentation in the nucleus seems to point away from the leading edge. × 6500.


COELOMIC
EPITELIUM
PGC
PGC

ment. In the 4–8-somite period of development, a few PGCs also were detected in the mesoderm at the base of the allantois. Although some have interpreted such evidence as indicating that PGCs originate in the endoderm, it should be emphasized that it indicates only that the PGCs in the endoderm of presomite mouse embryos are the first to have histochemically detectable levels of alkaline phosphatase activity.

There were other observations in this study that could be interpreted as evidence favoring the suggestion that PGCs may originate in the mesoderm or ectoderm and become located secondarily in the endoderm. For example, the PGCs and the cells of the yolk sac endoderm are quite different morphologically at the electron microscopic level. The endoderm cells have microvilli on their luminal surface, contain substantial amounts of endoplasmic reticulum and lipid and are connected to each other by adluminal junctions. PGCs do not reach the luminal surface, lack microvilli and junctions, have sparse amounts of endoplasmic reticulum and lipid, but have more densely staining cytoplasm than the endoderm cells. Although it might have been possible that PGCs acquired these special features soon after their differentiation, it seems less likely that they also could have discarded surface specializations, such as microvilli and junction, and eliminated some cytoplasmic organelles, such as endoplasmic reticulum and lipid droplets, during the same short time span. Substantial lysosomal activity would be necessary for the removal of membranes and organelles, but lysosomes are seldom seen in PGCs in the yolk sac endoderm. On the other hand, it was observed in this study that PGCs are rather similar in fine structure to mesodermal cells adjacent to the yolk sac endoderm. Also, PGCs often are enclosed incompletely by the endodermal cells and may exhibit small pseudopodal processes extending into the mesoderm. Some PGCs were seen even to lie between the endoderm and mesoderm, as noted in later stages by other workers (Spiegelman and Bennett, 1973). Furthermore, the endoderm is not separated from the mesoderm by a basal lamina at this time and is freely accessible to cells from the mesoderm.

Studies on the origin of the definitive endoderm in mammalian embryos suggest that the primitive ectoderm contains prospective endodermal cells (Grobstein, 1952). It has been proposed that the prospective endodermal cells migrate from the primitive ectoderm into the endoderm during the time between the appearances of the primitive streak and the head-fold (Levak-Švajger and Švajger, 1974). It was observed in this and previous studies (Mintz and Russell, 1957) that at comparable stages, 20–100 alkaline phosphatase-positive PGCs are first found scattered throughout part of the endoderm associated with the primitive streak. They may well have migrated from the primitive endoderm. The pattern of distribution of PGCs certainly seems more compatible with an immigration of PGCs or their precursors than an *in situ* origin. Since increases in alkaline phosphatase activity can be induced in other cells by a variety of physiological and pharmacological influences (Kaplan, 1972; Moss, 1974), the appearance of this enzyme on PGCs may thus represent their response to the new environment of the endoderm.

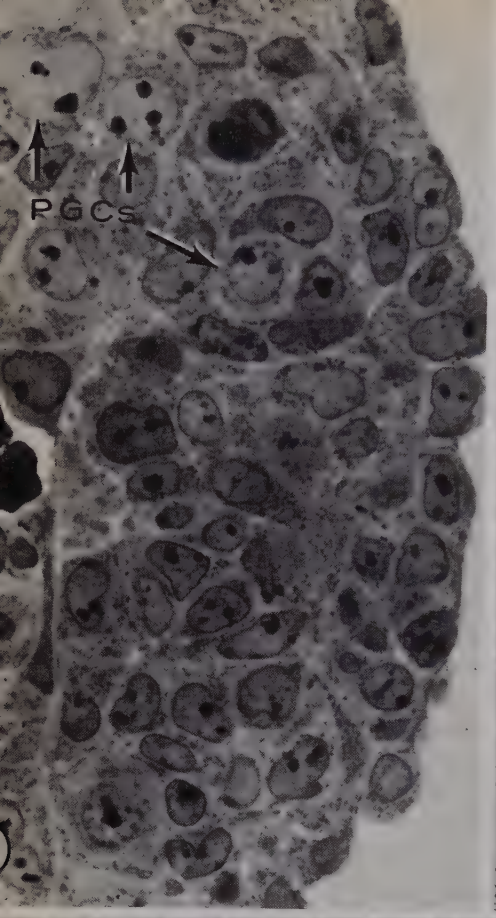
Although the preceding discussion favors the premise that mammalian PGCs arise elsewhere than in the endoderm, it leaves open the questions of how and where this occurs in the embryo. Two broad hypotheses have been proposed to explain the origin of PGCs. One hypothesis suggests that PGCs arise during development, differentiating from somatic cells (Nieuwkoop, 1949). The other, the so-called germ plasm hypothesis, suggests that the germ cells are continuous between successive generations, with germ plasm, the agent which determines PGCs, being transmitted through the gametes

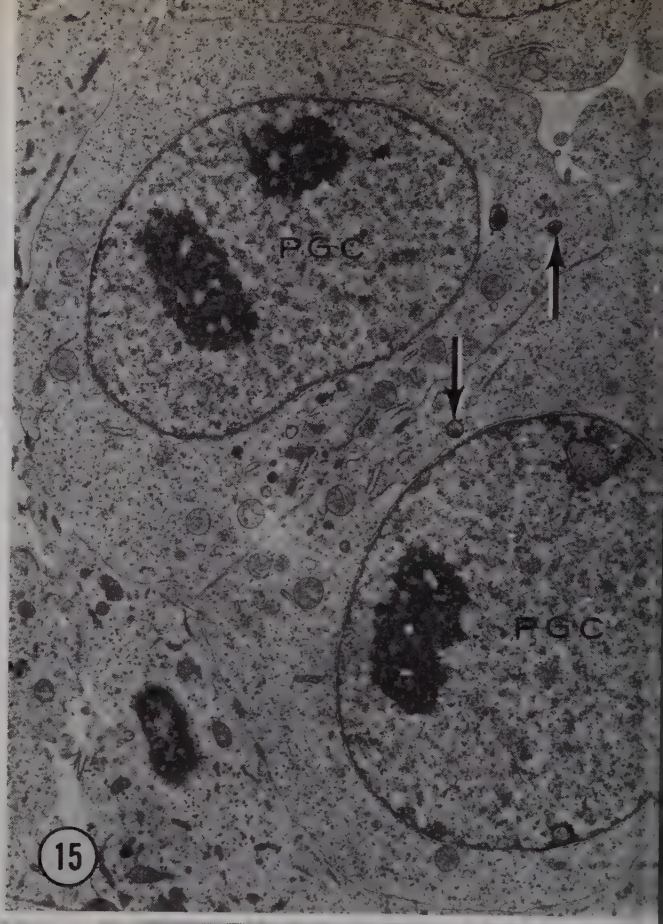
and into the embryo (Nussbaum, 1880; Weismann, 1892). Until recently, the germ plasm hypothesis has been considered only in relation to invertebrate and nonmammalian vertebrate germ cell determination (Beams and Kessel, 1974; Eddy, 1975). Because of similarities in form and distribution of the presumed germ plasm in those animals and nuage present in mammalian germ cells, it has been suggested that nuage may serve a similar role in mammalian germ cell determinations (Eddy, 1974). Dense, fibrogranular bodies representing the nuage have been observed in PGCs of the mouse (Spiegelman and Bennett, 1973) and of the rat (Eddy, 1974; Eddy and Clark, 1975). Evidence indicating that PGCs originate in the endoderm in this study would have favored the somatic cell hypothesis, while evidence that the PGCs originate elsewhere leaves open the possibility that either hypothesis may be true. Further work will be necessary to determine the time, place and means of initiation of the germ cells line in mammals and to learn which of these hypotheses applies.

Associations of PGCs. The germ cell line is rather unusual in that it does not exist as a discrete tissue but instead resides within various other tissues. It changes its residency several times during its life history. After being incorporated into the hindgut from the yolk sac, it moves through the dorsal mesentery to settle in the sex cords in the gonadal ridges. Except for the period of active migration through the dorsal mesentery, the PGCs are closely associated with epithelia. There is no direct evidence to explain this apparent dependence upon somatic cells (Zamboni and Merchant, 1973), but observations in this study and in others allow some suggestions to be made.

PGCs can first be recognized in the yolk sac endoderm, a tissue which would appear to be quite unrelated to the function of the germ cell line. However, it may be of advantage to the PGCs to be shunted to the yolk sac endoderm during the early part of development, away from regions of the embryo undergoing intense proliferation and extensive morphogenesis. This also might allow the germ cell line to be initiated rather early in development, before many other cell lines are well established and while some cells of the embryo still retain properties of the germ cells which produced the embryo. Another advantage may be nutritional, for although the PGCs in the yolk sac endoderm have only a modest number of organelles, the surrounding yolk sac cells are well equipped and have the morphological characteristics of metabolically active cells (Padykula *et al.*, 1966; Lambson, 1966; King and Enders, 1970; Haar and Ackerman, 1971). Since the chorioallantoic circulation is not established at this time, the yolk sac endoderm is probably the main source of nutrition to the embryo. Indeed, the endodermal cells of the yolk sac are known to absorb exogenous materials injected into the uterine lumen (Everett, 1935; Lambson, 1966; Beck *et al.*, 1967; Carpenter and Ferm, 1969; King and Enders, 1970; Haar and Ackerman, 1971) and may absorb the endogenous products of the uterine glands (Chiquoine, 1957). Also, the alkaline phosphatase localized on the microvilli of the cells in this study may be indicative of membrane transport, since it has been associated with transport in other microvillous cells (Kaplan, 1972; Russell *et al.*, 1972). Thus, the PGCs are situated particularly well to benefit from this activity and to derive nutrients from the endodermal cells.

During the next part of their life history, the PGCs become associated with the hindgut epithelium, probably as a result of morphogenetic events in which the portion of the yolk sac endoderm containing the PGCs becomes incorporated into the developing gut. This is a rather efficient means of transportation for the PGCs and has the advantage of placing them in the close vicinity of the regions where the gonadal ridges will soon form. Also, as was true


PGCs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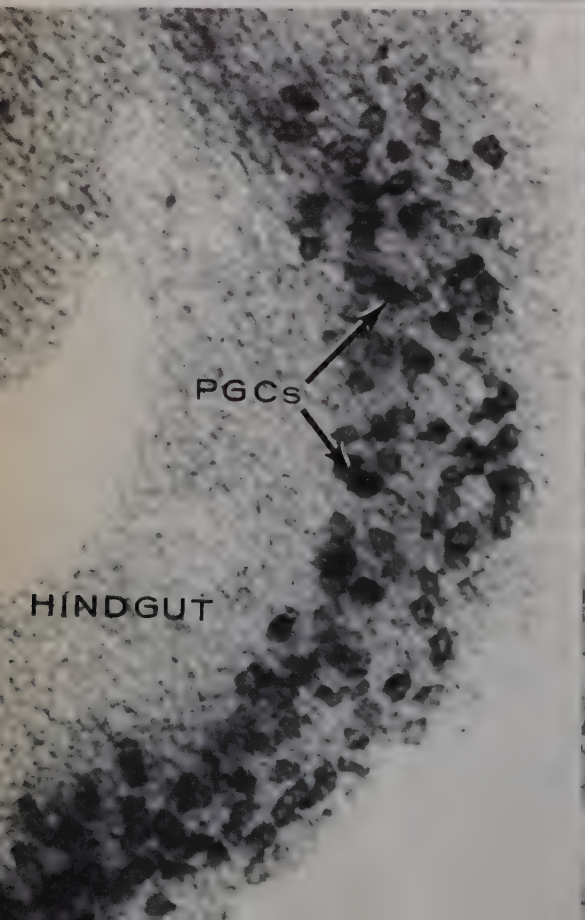
PGCs
HINDGUT


PGCs

before, while the cytoplasm for the PGCs is not highly specialized, that of the surrounding epithelial cells is well supplied with the components necessary for absorption or synthesis.

Soon after the connection between the gut and the yolk sac is closed and the vascular system becomes well established, the PGCs leave the gut epithelium, perhaps for a more desirable environment. Although the PGCs are not closely associated with any particular cell type in the dorsal mesentery, they appear to be in a well-vascularized, highly nutrient area (Zamboni and Merchant, 1973). They lie between the aorta and the coelomic epithelial cells, whose differentiated morphology suggests that they may be synthesizing nutrients or absorbing them for the coelom. Since the PGCs are never more than a few cells away from these areas, the somatic cells may be capable of maintaining the PGCs without close associations with them. Subsequently, the PGCs migrate to the gonadal ridges and eventually become surrounded by Sertoli cells or follicle cells. Although this is beyond the time period covered in the present study, it is of interest that these epithelia are believed to serve to modify or maintain the environment of the developing gametes (Biggers, 1972; Fawcett, 1973). It seems likely that these or similar roles are involved in the interrelationship between somatic cells and germ cells throughout the life history of the germ cell line.

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FIG. 14. Light micrograph of the gonadal ridge of a day-12 embryo. PGCs are seen as large, rounded and lightly staining cells lacking a rim of heterochromatin around the nuclear envelope. × 830.

FIG. 15. In an electron micrograph of a gonadal ridge of a day-12 embryo, the PGCs lack migratory features and usually exhibit a rounded nucleus but still may be identified by the presence of dense-cored vesicles (arrows). Focal junctions are present between adjacent germ cells and between germ cells and adjacent somatic cells. × 5300.

FIG. 16. A preparation of the hindgut region of a day-9 embryo that was fixed, reacted for alkaline phosphatase and then compressed between a slide and coverslip. The PGCs are contained for the most part within the gut epithelium. × 400.

FIG. 17. PGCs are seen within the gonadal ridge of a day-12 embryo that was compressed then fixed and reacted for alkaline phosphatase. The PGCs are closely associated in stacks. × 2500.

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Development of the Small Intestine in the Hypophysectomized Rat

I. Growth, Histology, and Activity of Alkaline Phosphatase, Maltase, and Sucrase

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Rats hypophysectomized at 6 days of age continue to grow but at a subnormal rate. At 24 days, when maturation of the intestinal epithelium normally culminates, the intestine is disproportionately small. The crypts are shallow and the mitotic rate low. The villi are short, and they fail to achieve the broad, leaflike form found in controls. The absorptive cells acquire a deep subnuclear zone, and their surfaces apparently cease to carry on pinocytosis. Rough endoplasmic reticulum is however sparse, and the Golgi complexes are small and atypical in structure. Duodenal alkaline phosphatase remains at the low level characteristic of the neonatal intestine. Sucrase activity appears in the jejunum, and maltase activity increases slightly, but both activities are less than a third of those in intact animals at 24 days. If the pituitary is removed later than 6 days, enzyme activities are higher than after early ablation, but they remain deficient even when the operation is performed at 16 days.

INTRODUCTION

The intestinal lining of the rat at birth is formed into villi covered by functionally competent epithelial cells that are segregated from a population of proliferative cells restricted to shallow crypts. The villous epithelium differs from that of the adult in numerous respects that are related to the nature of the diet. After suckling has begun, the epithelial cells have a vacuolated appearance due to the ingestion of lipids and proteins into intermicrovillous infoldings that communiate with a series of tubules and cisternae in the supranuclear zone (Clark, 1959; Cornell and Padykula, 1969; Vollrath, 1969). Concomitantly the epithelial cells are able to absorb antibodies (Halliday, 1955; Kraehenbuhl and Campiche, 1969; Rodewald, 1973). The activity and distribution of enzymes associated with the microvillous membrane also reflect the functional demands of the nursling period. Lactase activity is high (Doell and Kretchmer, 1962; Rubino, *et al.*, 1964; Goldstein *et al.*, 1971), but maltase is low and sucrase virtually absent (Rubino *et al.*, 1964; Doell and Kretchmer, 1964; Herbst and Koldovsky, 1972). Aminopeptidase activity is low (Noack *et al.*, 1966; Vollrath, 1969), and the highly active alkaline phosphatase peculiar to the duodenum has not appeared (Moog and Yeh, 1973).

The shift from the infantile to the mature state of the epithelium is in most respects not gradual but is rather restricted to a relatively short critical period that is controlled by the adrenal cortex. Such endocrine regulation, first demonstrated systematically in the mouse (Moog, 1953), has since been shown to occur also in the rat, in which the critical period extends from about the 19th to the 23rd day. Administration of glucocorticoids to the suckling rat not only induces precocious appearance or increase of alkaline phosphatase (Halliday, 1959) and α-disaccharidases (Doell and Kretchmer, 1964; Herbst and Koldovsky, 1972; Galand and Forstner, 1974) but also brings the capacity for ingestion of macromolecules to a premature close (Clark, 1959; Halliday, 1959; Jones, 1972). Adrenalectomy in the suckling rat prevents the occurrence of various maturational events at the appropriate time (Koldovsky *et al.*, 1965; Daniels *et*

al. 1973), and Daniels *et al.* (1972) have shown that the normal onset of closure is accompanied by a marked increase of plasma corticosterone levels.

That the activation of the adrenal cortex at the critical period is due to pituitary stimulation has been inferred from the fact that premature intestinal maturation may be elicited in the mouse either by stress or by administration of ACTH (Moog, 1953). This inference obviously requires direct confirmation by ablation of the pituitary in the neonatal stage. Such an operation has been performed on rats by Walker *et al.* (1950), who were able to keep some hypophysectomized animals alive for more than 2 months, despite high mortality. In animals deprived of the pituitary gland, the growth of stomach and intestine was retarded as much as that of the body as a whole, but no histological observations were reported (Walker *et al.*, 1952). The thyroid and adrenal failed to gain any weight after the removal of the pituitary (Walker *et al.*, 1950). Hence the intestine of the hypophysectomized suckling might suffer not only from lack of glucocorticoids and of growth hormone but from lack of thyroxine as well.

Although thyroxine and a pituitary factor that may be prolactin are essential for normal intestinal epithelial maturation in the chick embryo (Moog, 1961; Hinni and Watterson, 1963; Hart and Betz, 1972), evidence for the involvement of hormones other than glucocorticoids in intestinal development in suckling mammals has been marginal. Growth hormone has been shown to regulate mitotic rate in the crypts of the adult rat intestine (Leblond and Carriere, 1955), and brief reports have indicated that exogenous thyroxine can cause premature cessation of macromolecular uptake (Chan *et al.*, 1973) and elicit precocious increase of α-disaccharidases (Koldovsky *et al.*, 1974) in intact animals. To gain a more systematic understanding of the role of the thyroid hormone and others, we have begun a series of studies on the effects of hypophysectomy and thyroidectomy on intestinal maturation. The results have already demonstrated that the normal decrease of lactase activity is dependent on thyroxine (Yeh and Moog, 1974). This paper and the one following (Yeh and Moog, 1975) further detail our findings on the growth and differentiation of the small intestine in hypophysectomized rats. Some of these results have been presented in abstract (Yeh and Moog, 1973).

MATERIALS AND METHODS

Animals. The rats used are of the NLR strain, obtained from the National Laboratory Animal Company, Creve Coeur, Mo. They are fed Purina Laboratory Chow. Adults are mated in our laboratory, and litters are reduced to ten on the 2nd day after birth. The young remain with the mother to the age of 28 days. They were not fasted before being killed.

Surgical methods. Anesthesia is effected by exposing animals younger than 16 days to −15°C for about 30 min. The resulting anesthesia lasts 10–15 min. Nembutal (0.01 mg/g body weight) is used for animals of 16 days.

Hypophysectomy is carried out by the parapharyngeal approach, as modified by Walker *et al.* (1950) for very young rats. The operation takes 8–10 min, and ordinarily very little bleeding occurs. Sham-operated animals are subjected to the same procedure, except for removal of the gland. All experiments included hypophysectomized and intact and/or sham-operated controls within a single litter. After sacrifice of the animals, the completeness of hypophysectomy was evaluated by inspection of the pituitary fossae in serial sections. If any fragment of adenohypophysis was found, the thyroid was examined for degree of atrophy, and the adrenals were weighed to determine if weight was subnormal.

Of 395 animals hypophysectomized at 6 days of age, 150 (38.0%) died before 24

days; the majority of these failed to nurse and succumbed within 4 days after operation. The survivors included 160 (40.5%) that proved to be completely hypophysectomized, and 85 (21.5%) that were incompletely hypophysectomized. Data obtained from the latter group were excluded from the results reported.

Collection of tissue. The rats were sacrificed by decapitation at 20, 24, or 28 days. The small intestine was removed and cleaned of adhering tissue. A 3-cm segment extending from the pylorus (duodenum) and a 5-cm segment centered midway along the length of the tube (jejunum) were cut out, stripped of content by gentle pressure, weighed on a Roller–Smith torsion balance, and stored in 0.15 *M* NaCl at −24°C. The stomach, the remainder of the small intestine, and the large intestine and cecum were also weighed, minus contents in all cases. The adrenals were also weighed routinely. The heads, tracheas with adherent thyroids, and adrenals were fixed in Bouin's fluid for histological examination.

Preparation for microscopy. Small segments of intestine taken near the duodenojejunal boundary were used for all structural studies. For light microscopy, specimens were fixed in Bouin's fluid or in cold 80% alcohol, subsequently embedded in paraffin, and sectioned at 5 μm. Hematoxylin and eosin were used for routine histological examination, and for cell counts. The periodic acid–Schiff reaction (Pearse, 1968) was used with alcohol-fixed material to examine the distribution of polysaccharides, and Gomori's cobalt–sulfide technique (Pearse, 1968) was used to localize alkaline phosphatase activity. For all histochemical studies, sections from hypophysectomized and control litter mates were affixed to the same slide.

For electron microscopy, tissue was trimmed and fixed overnight in cold 3% glutaraldehyde in 0.1 *M* phosphate buffer, pH 7.4. It was then postfixed in 1% osmium tetroxide in 0.1 *M* phosphate buffer, pH 7.4, for 3 hr and embedded in Epon-812. Thick sections (1–2 μm) of entire villi were cut and stained with toluidine blue to orient and trim blocks. Thin sections (600–900 Å) were poststained with uranyl acetate followed by lead citrate, and examined in a Hitachi HU-11C electron microscope.

For examination in the scanning electron microscope, intestinal segments were flattened on paraffin plates with the aid of dissecting needles. The epithelial surface was washed by a stream of normal saline, then fixed as for transmission electron microscopy. Fixed tissues were dehydrated in acetone and transferred to liquid Freon for critical point drying (Smith and Finke, 1972); because of a shortage of liquid Freon, however, tissues from 6-day-old rats had to be air dried. After drying all tissues were coated with chromium in a Kinney vacuum evaporator and viewed in a Cambridge scanning electron microscope.

Biochemical methods. Collected segments were thawed and homogenized in 0.15 *M* NaCl in a Ten Broeck grinder. Because of the thin and fragile nature of the submucosal layers in all stages examined, whole intestinal wall was used rather than scraped mucosa.

Alkaline phosphatase activity was determined with both phenylphosphate (PhP; Schwarz/Mann) and β-glycerophosphate (βGP; Sigma) as substrates by methods that we have previously applied to the young rat intestine (Moog and Yeh, 1973). The reaction mixture consisted of 1.0 ml of 120 m*M* substrate solution and 1.5 ml of 200 m*M* carbonate–bicarbonate buffer containing 16 m*M* $MgCl_2$. Buffer pH was 9.8 for PhP, 9.4 for βGP. After the mixture was warmed at 37.5°C, 0.5 ml of duodenal homogenate was added. Tissue concentration was 0.1 or 0.25 mg/ml for PhP, 0.5 mg/ml for βGP. When PhP was the substrate, released phenol was determined by the King–Armstrong (1934) technique; with βGP the Fiske–Subbarow (1925)

ethod for phosphorus was used. Colors ere read in a Klett–Summerson colorime- r with a 540-nm filter for phenol and a 60-nm filter for phosphorus. All assays ere run in duplicate.

Maltase and sucrase activities were easured by Dahlqvist's (1968) technique. or maltase, the reaction mixture con- sted of 56 m*M* maltose (Sigma) in 0.1 ml f 100 m*M* maleate buffer at pH 5.8 plus .1 ml of a tissue homogenate containing 1 ng/ml; for sucrase, conditions were the ame except that the sucrose (Sigma) con- entration was 140 m*M* and the tissue ho- nogenate contained 5 mg/ml. After 60-min ncubation at 37.5°C, the reaction was topped by addition of 2 ml of freshly nixed Tris–glucose oxidase prepared from Glucostat reagent (Worthington Biochemi- al Company). Color was developed at 7.5°C for 60 min and read in a Coleman Junior IIA spectrophotometer at 430 nm. Each test was run in duplicate. Standards ontaining 20–100 μg of glucose were run n parallel with each set of determina- ions.

Protein content was measured by the echnique of Lowry *et al.* (1951). Results ere evaluated statistically by Student's *t* est or by Wilcoxon's signed-ranks test Snedecor, 1961) where appropriate.

RESULTS

Growth of Body and of Gastrointestinal Tract

Ablation of the hypophysis at 6 days etards but does not arrest growth, the perated animals gaining weight steadily p to 24 days, with no further increase Fig. 1). The gastrointestinal tract also con- inues to grow. When hypophystectomy is erformed at 6 days, the weight of the mall intestine at 20 days is more than wice that at 6 days, but no further growth ccurs up to 28 days (Fig. 2). After hypoph- sectomy at 10 days, the weight of whole ut (stomach, cecum, and small and large ntestine) increases slightly up to 24 days,

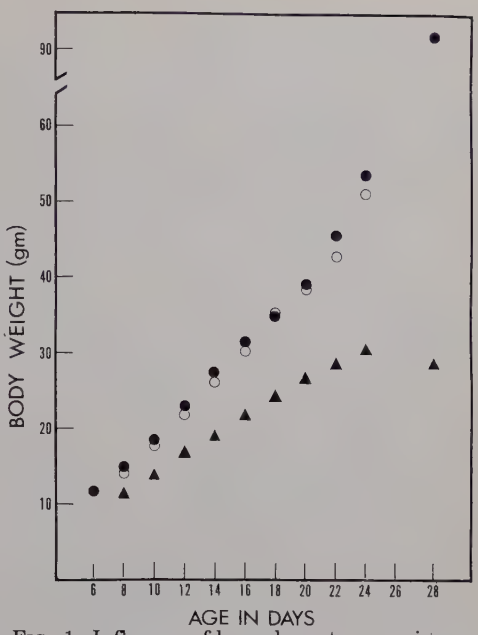


FIG. 1. Influence of hypophysectomy on increase of body weight in rats. (●), Intact rats; (○), sham-operated at 6 days; (▲), hypophysectomized at 6 days. Each symbol represents the mean weight of ten animals.

but that of intestine alone scarcely increases at all; operation at 14 days arrests gut growth completely. Between 16 and 24 days the gut grows more rapidly than the body as a whole, but this is not true in hypophysectomized animals, in which relative weights remain at or below the level in normal animals between 6 and 16 days (Fig. 2). Thus in rats hypophysectomized at 6 days, the relative weight of the small intestine at 24 days is 22.4 ± 0.74 mg/g, compared with 38.5 ± 0.80 in intact rats at 24 days and 30.4 + 0.52 in intact rats at 6 days; the difference between the first value and either of the others is significant ($P < 0.005$). The absolute and relative growth of the whole gut parallels that of the small intestine alone.

Structure of the Intestinal Lining

At 6 days the villi at the duodenal-jejunal boundary are relatively short and the crypts shallow. In the ensuing 18 days the crypts more than double in depth, and

the villus height increases substantially (Table 1). The villi also change in shape, being tongue-like at 6 days but subsequently broadening in one dimension (Fig. 3). In 24-day-old animals hypophysectomized at 6 days, the villi are slightly shorter than in 6-day-old controls; the crypts are deeper, but do not attain the normal 24-day depth. The villi fail to broaden (Fig. 3), and the mitotic index does not undergo the normal increase in hypophyseoprivic young (Table 1). Our findings on intestinal growth in intact animals are in good agreement with those of Herbst and Sunshine (1969) on Wistar rats.

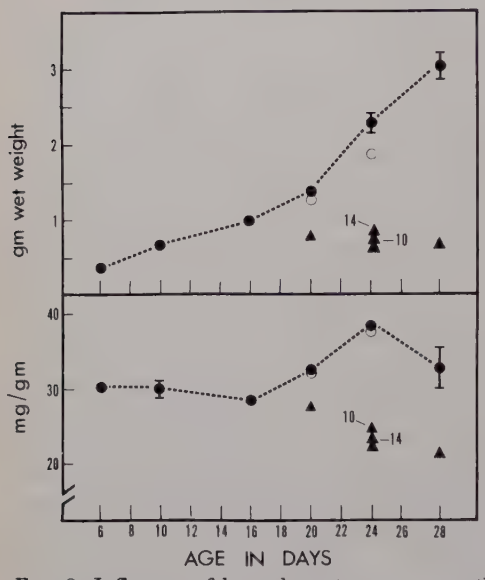


FIG. 2. Influence of hypophysectomy on growth of the small intestine in rats. Above: Weight of whole small intestine; below: Relative weight of intestine as milligrams per gram body weight. Symbols as for Fig. 1, except that at 24 days two of the solid triangles represent mean weights of animals hypophysectomized at 10 or 14 days, as indicated. Each symbol is the average of five to nine intestines, except for 16 days (two intestines). SEM is indicated by vertical lines when larger than the symbol used. For tests of significance, see text.

At 6 days the epithelial cells are relatively broad and short, with the nuclei situated close to the basement membrane (Fig. 4). They display characteristics that have been amply described (Clark, 1959; Cornell and Padykula, 1969; Worthington and Graney, 1973). Most conspicuous is the vacuolization of the apical cytoplasm (Fig. 5). The larger vacuoles no doubt contain material engulfed by pinocytotic infoldings between the bases of the microvilli (Figs. 5 and 8). Lysosome-like bodies are seen occasionally. Parallel arrays of rough endoplasmic reticulum are abundant in some cells (Fig. 5), sparse in others, and are not predominantly aligned along the surfaces of mitochondria. Flattened cisternae are rarely seen in the supranuclear zone in which the Golgi complex is found at later stages.

By 24 days the cells have acquired most of the characteristics of the adult stage

TABLE 1

MITOTIC INDICES AND DIMENSIONS OF VILLI AND CRYPTS IN INTACT (I) RATS AND RATS HYPOPHYSECTOMIZED (H) AT 6 DAYS

Group	Age (days)	Number	Villus height (number of cells)[b]	Crypt depth (number of cells)[b]	Mitotic index[a]
Intact	24	7	78.5 ± 2.3	19.1 ± 0.37	4.05 ± 0.13
			$P < 0.001$[c]	$P < 0.001$	$P < 0.001$
Hypex	24	7	51.7 ± 0.60	11.3 ± 0.60	2.70 ± 0.06
			$P < 0.05$	$P < 0.005$	$P < 0.025$
Intact	6	6	57.1 ± 0.8	8.8 ± 0.26	2.48 ± 0.04

[a] Mitotic figures from prophase to telophase were counted in longitudinally sectioned crypts. At least 50 crypts, totalling 2000–3000 cells, were counted in each animal.

[b] Cells were counted on one side of a longitudinally sectioned villus or crypt; ten villi and ten crypts were counted in each animal.

[c] Each P value refers to the difference between the value above and below it in the column.

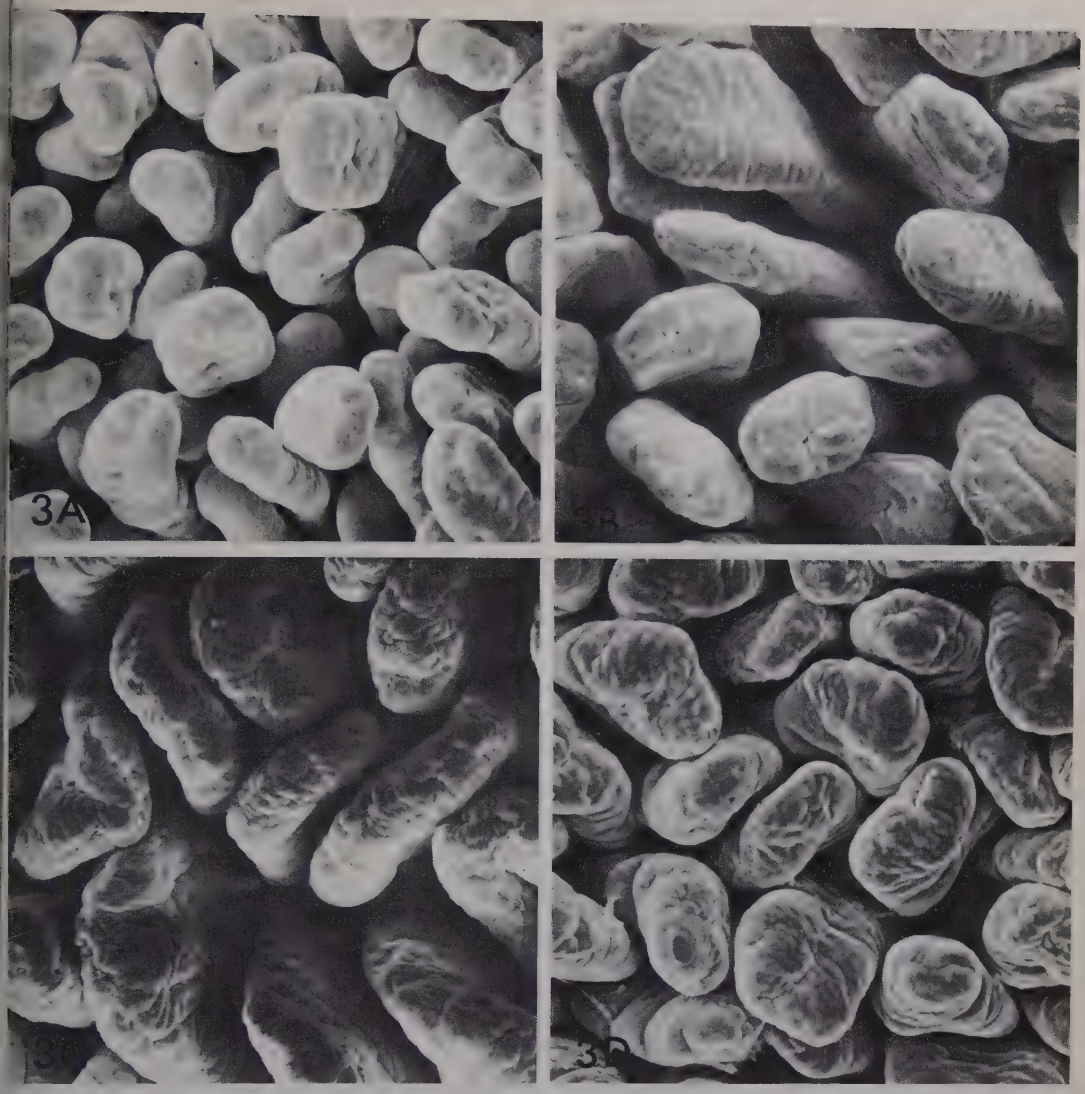


FIG. 3. Villi in the distal portion of the duodenum, as seen in the scanning electron microscope. (A), 6-day-old rat, intact; (B), 14 days, intact; (C), 24 days, intact; (D), 24 days, hypophysectomized at 6 days. 180×.

(Clark, 1959; Trier, 1968). Subjacent to the brush border there is a well-developed terminal web that is free from inclusions (Fig. 6). The apical cytoplasm contains only a few small vesicles, and the membrane at the bases of the microvilli no longer forms deep invaginations. The Golgi complex, which has become conspicuous, includes numerous lamellar membranes surrounded by small vesicles. Rough endoplasmic reticulum is abundant and, in contrast to the 6-day stage, appears in long parallel arrays predominantly oriented close to the surfaces of mitochondria. A few lysosome-like bodies are present.

After hypophysectomy at 6 days, the epithelial cells at 24 days resemble those of intact controls of the same age more closely than they do those of 6-day-old animals (Fig. 4). Only small vesicles occur in the apical area, and the tubular invaginations of the surface membrane have largely disappeared (Figs. 7 and 9). The Golgi complex is smaller than in controls

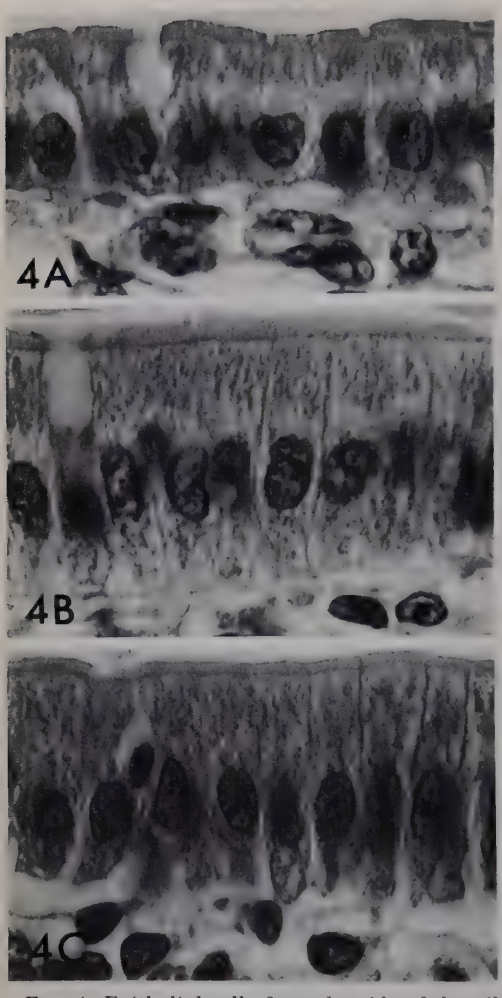


FIG. 4. Epithelial cells from the side of the villus, as seen in the light microscope. (A), 6-day-old rat, intact; (B), 24 days, intact; (C), 24 days, hypophysectomized at 6 days. 1150×.

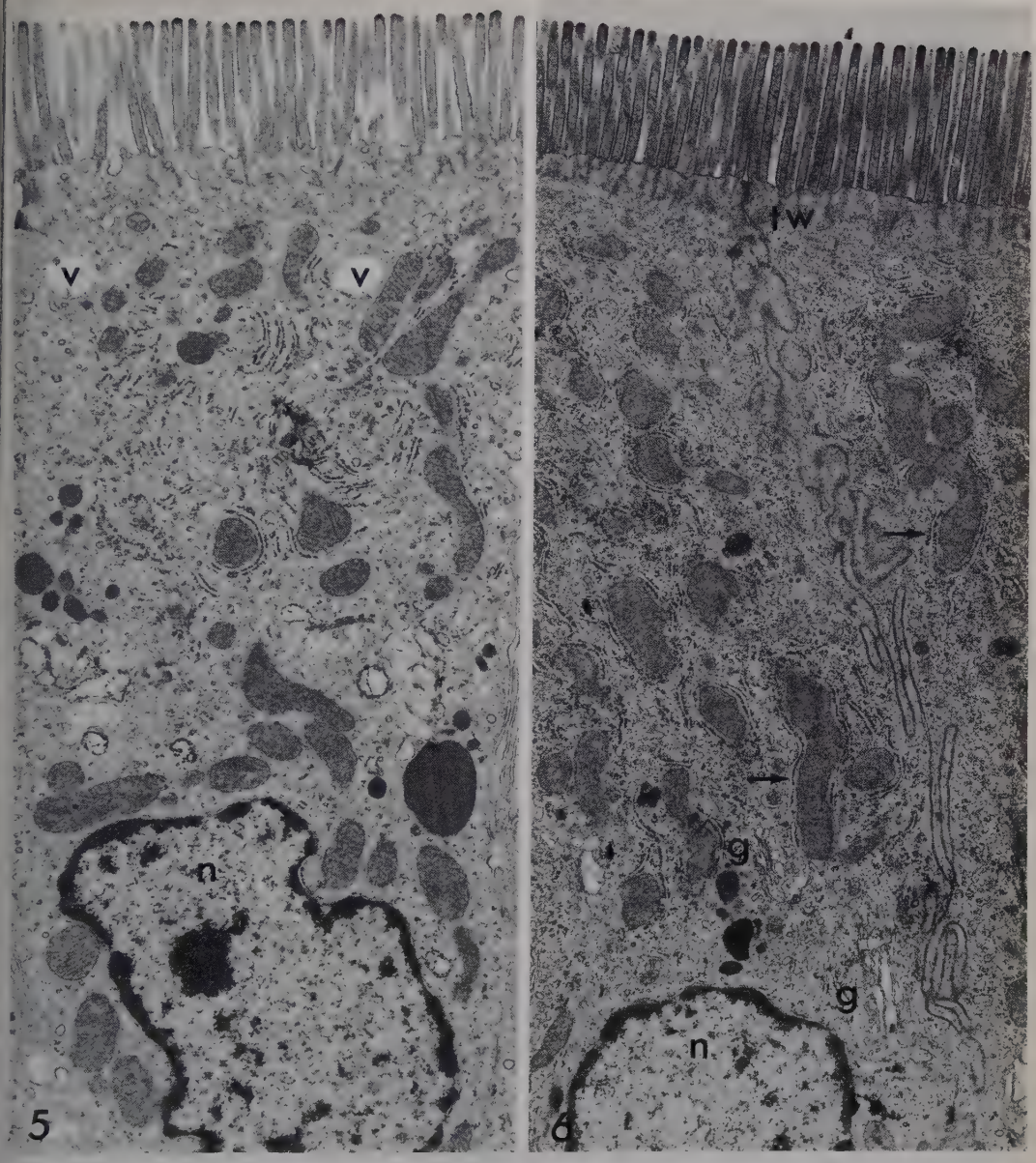
and generally lacks the typical organization of stacked membranes bordered by vesicles (see Yeh and Moog, 1975). There is no paucity of ribosomes, but rough endoplasmic reticulum is deficient in quantity, and such rough-surfaced cisternae as are seen are only infrequently associated with mitochondria. A broad basal cytoplasmic zone has appeared (Fig. 4).

The microvilli midway up the side of the villus are longer and narrower at 24 days than at 6 days, but the variation from villus to villus, and even from cell to cell, precludes accurate quantitation of these differences. Similar variation is also found in the hypophysectomized animals (Figs. 7A and B), in which the microvilli were in some cases as long as the longest found in controls. Very little glycocalyx is seen at any stage. Use of the periodic acid–Schiff technique, however, produced much heavier staining of the brush border at 24 days than at 6 days, and the hypophysectomized specimens were plainly deficient in stainability (see Yeh and Moog, 1975).

Enzyme Activities in the Intestine

Alkaline phosphatase in the duodenum. The effect of hypophysectomy on intestinal enzyme activities is shown in abbreviated form on Fig. 10 and in detail in Table 2. Duodenal phosphatase activity falls until 16 days, rises to a peak at 24 days, and then declines (Table 2, lines 1–7). The increase is much greater when PhP is used as substrate than when βGP is used (under optimal conditions for each), so that the ratio of PhP to βGP utilized rises from less than 1 at 6 days to more than 3 at 24 days (Table 2). This shift, which has been shown in the mouse to result from the appearance of a new family of phosphatase isozymes (Etzler and Moog, 1968), has been previously reported in the rat (Moog and Yeh, 1973).

When rats are hypophysectomized at 6 days, their duodenal phosphatase at 20 days, assayed with PhP, is at the minimal level normally reached at 16 days (Table 2, lines 4 and 8). By 24 days, however, it rises significantly ($P < 0.005$), although remaining far below the normal 24-day PhP peak (lines 6 and 9). When hypophysectomy is performed later than 6 days, phosphatase activities are progressively higher (lines 9–12), but even if the operation is delayed until 16 days, the PhP activity attained at 24 days is less than 30% of the normal (lines 6 and 12). Regardless of the time of hypophysectomy, activity with both substrates is equally affected, so that the PhP/βGP ratio does not rise much above unity. Between 24 and 28 days, specific activities with both substrates remain at



FIGS. 5 and 6. Electron micrographs showing the supranuclear zone of intestinal absorptive cells halfway up the side of the villus. 9100×. Fig. 5, 6-day-old rat. The apical surface shows numerous infoldings. RER is abundant but is not predominantly associated with mitochondria. The large vesicles (v) are presumably engaged in the breakdown of material taken in by endocytosis. The zone above the nucleus (n) is highly vacuolated, but typical Golgi membranes are not seen. Fig. 6, 24-day-old rat, intact. An inclusion-free terminal web (tw) is present, and the apical surface is uninterrupted by tubular invaginations. RER tends to border the mitochondria (arrows). Typical Golgi complexes (g) are evident.

about the same level in rats hypophysectomized at 6 days (lines 9 and 13). Protein content rises only to approximately the 20-day level in hypophyseoprivic animals but declines to the normal extent between 24 and 28 days. Histochemical staining showed that phosphatase activity is localized in the brush border of villus epithelial cells under all conditions of study.

Maltase and sucrase in the jejunum. Di-

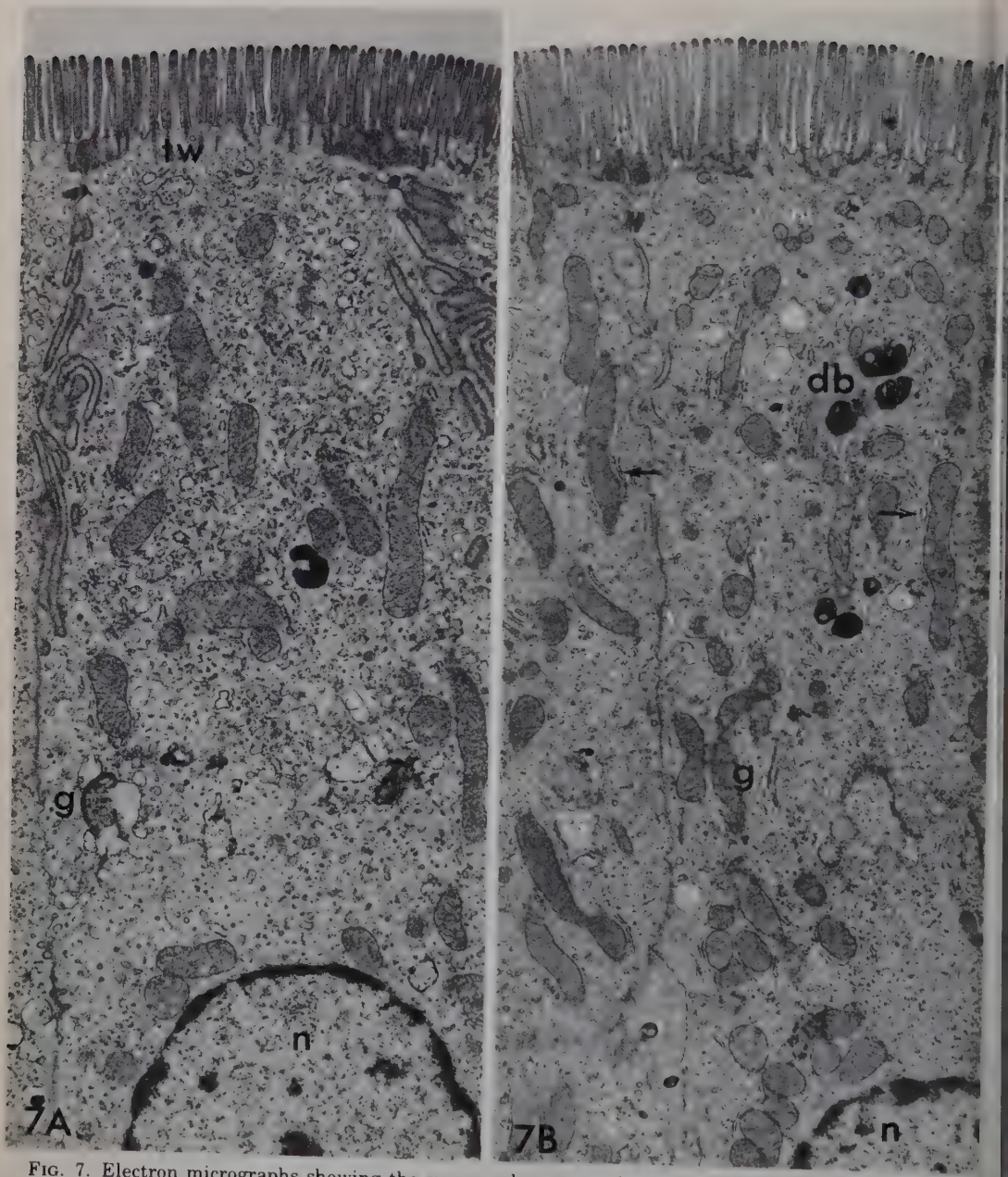


FIG. 7. Electron micrographs showing the supranuclear zone of intestinal absorptive cells halfway up the side of the villus. 9100×. 24-day-old rats hypophysectomized at 6 days. (A), The terminal web (tw) contains only an occasional small invagination. The apical area contains numerous small vesicles but no large ones. Only short lengths of parallel RER membranes are seen. Golgi membranes (g) are short and are associated with exceptionally large vacuoles. (B), The apical surface is less regular than in (A). There are a few vesicles of moderate size, with a variety of included matter, and several dense bodies (db). RER is sparse but is sometimes oriented parallel to mitochondrial surfaces (arrows). Golgi membranes (g) have little associated vesiculation.

TABLE 2

ENZYME ACTIVITIES IN THE INTESTINE OF INTACT (I) RATS AND RATS HYPOPHYSECTOMIZED AT 6, 10, 14 OR 16 DAYS (H), AND SHAM-OPERATED AT 6 DAYS (S)[a]

Line	Group	Age at determination (days)	No.	Duodenum: Alkaline phosphatase				Jejunum: Maltase and sucrase			
				Protein (μg/mg)	PhP (μmol of substrate mg of protein/hr)	βGP	PhP/βGP[b]	Protein (mg/mg)	Maltase (μmol of substrate/ mg of protein/hr)	Sucrase	M/S[b]
1	I	6	6	122 ± 3.7[c]	99 ± 7.6	102 ± 7.0	0.89	131 ± 6.0	2.13 ± 0.10	—	
2	I	10	6	127 ± 2.0	81 ± 5.0	90 ± 5.6	0.90	137 ± 4.3	1.78 ± 0.14	—	
3	I	14	6	140 ± 4.9	73 ± 8.2	80 ± 11	0.94	144 ± 6.2	1.67 ± 0.14	—	
4	I	16	6	139 ± 4.7	62 ± 9.6	67 ± 11	1.05	145 ± 6.3	1.74 ± 0.20	—	
5	I	20	6	155 ± 4.9	135 ± 19	95 ± 7.4	1.40	161 ± 5.5	6.88 ± 0.63	1.42 ± .18	4.5
6	I	24	15	163 ± 3.0	680 ± 36	174 ± 10	3.71	150 ± 3.6	18.94 ± 0.06	3.90 ± .19	4.9
7	I	28	4	149 ± 5.2	459 ± 43	158 ± 13	2.89	139 ± 2.3	14.44 ± 1.76	2.65 ± .13	5.3
8	H6	20	8	147 ± 3.5	59 ± 3.6	57 ± 4.6	1.03	155 ± 3.0	1.84 ± 0.09	0.23 ± .04	9.9
9	H6	24	9	156 ± 2.8	104 ± 10	85 ± 9.0	1.24	152 ± 2.8	3.39 ± 0.44	0.80 ± .16	4.8
10	H10	24	7	151 ± 7.1	123 ± 11	116 ± 12	1.13	158 ± 8.2	3.44 ± 0.35	0.95 ± .16	4.1
11	H14	24	5	152 ± 2.8	149 ± 12	139 ± 14	1.09	148 ± 6.8	5.43 ± 0.49	1.36 ± .16	4.1
12	H16	24	5	151 ± 5.6	193 ± 19	166 ± 10	1.17	138 ± 6.1	5.38 ± 0.56	0.97 ± .16	6.3
13	H6	28	5	137 ± 3.4	109 ± 18	99 ± 13	1.11	144 ± 4.3	4.80 ± 0.88	1.07 ± .24	4.9
14	S6	20	7	154 ± 5.0	119 ± 16	93 ± 6.6	1.26	150 ± 3.4	6.32 ± 1.40	1.19 ± .31	5.8
15	S6	24	6	165 ± 3.9	555 ± 44	114 ± 10	4.77	155 ± 1.5	16.47 ± 0.85	3.79 ± .13	4.8

[a] For assay conditions, see Materials and Methods. For appropriate tests of significance, see Results.

[b] Means of ratios of individual tests.

[c] Mean ± SEM.

saccharidase activity was assayed in the jejunum, where it reaches maximal levels in the mature rat intestine (Rubino *et al.*, 1964; Goldstein *et al.*, 1971). The pattern of maltase activity is similar to that of duodenal phosphatase, falling after 6 days and rising after 16 days to a peak at 24 days from which it declines (Fig. 10 and Table 2, lines 1–7). In animals hypophysectomized at 6 days, activity at 20 days is still at the normal 16-day level (Table 2, lines 4 and 8); a significant rise occurring between 20 and 24 days ($P < 0.001$, line 8 vs 9) apparently continues to 28 days, but the 24–28-day difference is not significant ($P > 0.10$, line 9 vs 13). When hypophysectomy is performed at 14 or 16 days, activity at 24 days is higher than after operation at 6 days ($P < 0.025$, line 9 vs 11 or 12).

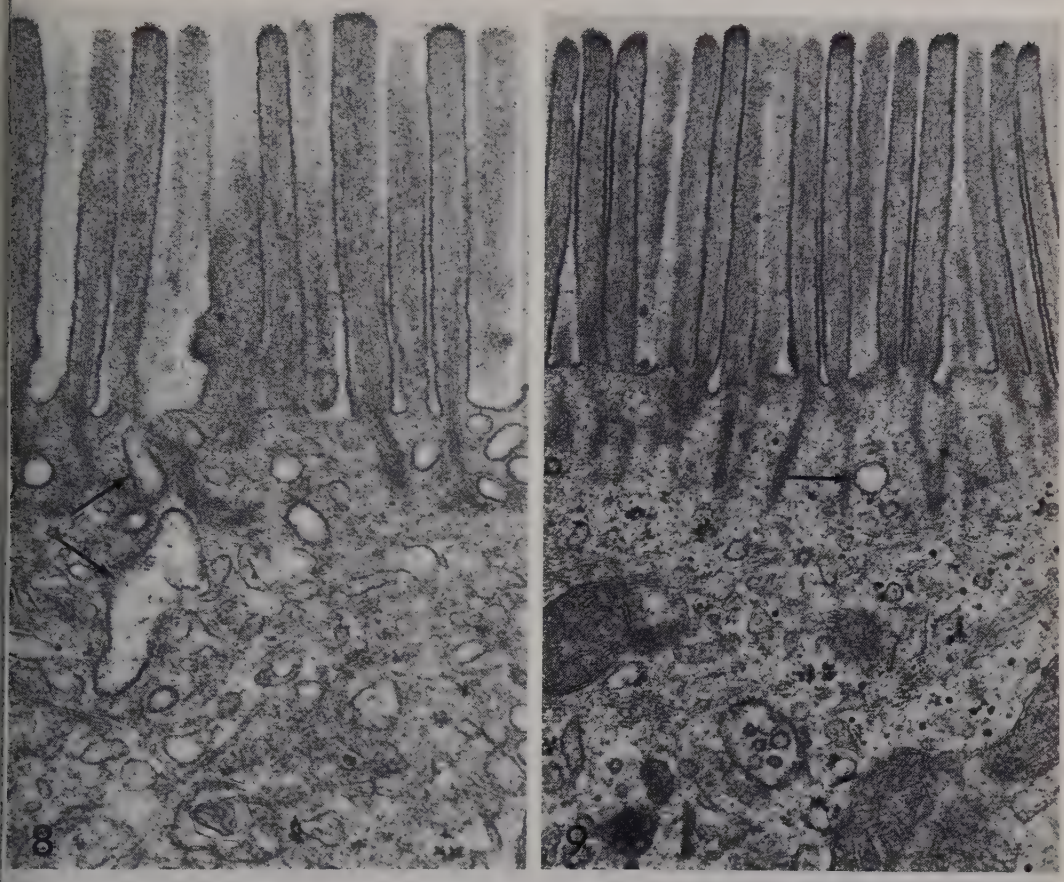
Sucrase first becomes measurable at 20 days, peaks at 24 days, and declines (Fig. 10 and Table 2, lines 5–7). After hypophysectomy at 6 days, sucrase activity also appears at 20 days but probably with some delay, since the ratio of maltase to sucrase averages 9.9, compared with a control ratio of 4.5 (Table 2, line 5). At 24 days, sucrase activity is significantly higher ($P < 0.001$, line 8 vs 9), and the level is sustained to 28 days. Hypophysectomy at 14 days, but not at 10 or 16 days, results in higher sucrase at 24 days than in 6-day operated animals ($P < 0.05$, line 9 vs 11).

DISCUSSION

The continued slow growth that we have observed after hypophysectomy at 6 days is in agreement with earlier demonstrations that growth in the suckling stage is partly independent of the pituitary. In all cases reported, growth does however slacken or stop in the 3rd or 4th week (VanEck and Freud, 1941; Walker *et al.*, 1952; Diamond, 1968). The increasing disparity in weight between controls and hypophysectomized rats in this period is partly due to the fact that the former begin to supplement the milk supply by eating adult chow. The concurrent growth failure of the latter, however, raises two questions: What supports the substantial, albeit subnormal, growth that occurs during the first 2 weeks after the operation? What causes the subsequent severe retardation of growth? The second question cannot be answered simply in terms of a diminished milk supply, for in the absence of the pituitary animals grow very little after weaning, even though surviving for a month on more on a soft diet (Asling *et al.*, 1952). Our observations indicating that the intestine of the hypophysectomized pup loses the capacity for endocytosis without gaining full use of the enzymatic mechanisms characteristic of the mature organ may provide a part of the explanation. The hypophyseoprivic animals may not have the means to digest and absorb any kind of food adequately.

In answering the first question raised above, one must rule out growth hormone and probably thyroxine as well. The possibility that prolactin may be implicated is suggested by its high titer in rat's milk (McMurty and Malven, 1974). Prolactin resembles growth hormone in structure (Bewley and Li, 1970) and has growth-promoting properties that have been demonstrated in a variety of animals (Riddle, 1963; Wallis and Dew, 1973). The decline in the prolactin content of milk beginning in the 3rd week (McMurty and Malven, 1974) might also be a factor in the subsequent cessation of size increase in hypophyseoprivic rats. In our laboratory we have however not been able to stimulate growth in hypophysectomized animals by daily administration of 50 or 500 μg of ovine prolactin from the 16th through the 23rd day.

After hypophysectomy at 6 days, the gut as a whole and the small intestine in particular continue to grow at about the same rate as the body up to 20 days, when the relative weights of the gastrointestinal structures are the same as at 6 days or slightly less. At the time when differential growth carries the relative weight of intestine (and of whole tract) to peak levels at



FIGS. 8 and 9. Electron micrographs showing the apical surface area of absorptive cells halfway up the side of the villus. 34,200×. Fig. 8, 6-day-old rat. Terminal web area contains numerous vesicles derived from the surface membrane. The large vesicle and tubular invagination indicated by arrows are probably connected. Fig. 9, 24-day-old rat hypophysectomized at 6 days. Terminal web includes only one vesicle (arrow) that might possibly have been derived from the surface.

24 days (Fig. 2), however, these organs not only fail to grow at an enhanced rate in hypophysectomized rats but actually lose weight. Thus in rats hypophysectomized at 6 days the small intestine weights 788 ± 40 mg at 20 days, compared with 631 ± 34 mg at 24 days ($P < 0.005$); the corresponding relative weights are 27.6 ± 0.80 vs 22.4 ± 0.74 mg ($P < 0.005$). The ability of the intestine to grow thus appears to be highly dependent on the hypophysis in the same critical period, beginning in the 3rd week, in which aspects of structural and biochemical differentiation go forward rapidly (Halliday, 1955; Clark, 1959; Rubino *et al.*, 1964; Williams and Beck, 1968; Moog and Yeh, 1973).

The heightened growth rate of the critical period is apparently not due to growth hormone, which reaches its lowest concentration at 20 days (Blasquez *et al.*, 1974). Specific tropic hormones probably play an important role, however, since glucocorticoids are essential to normal differentiation in this period (Moog, 1953, 1962; Koldovsky *et al.*, 1965; Daniels *et al.*, 1973), and thyroxine is also (Yeh and Moog, 1974, 1975). Cortisone or thyroxine alone, at dose levels that strongly elevate enzyme activities, do not affect intestinal growth in hypophysectomized pups, and growth hormone does not either (Yeh and Moog, 1975), but a combination of hormones might be effective. The doubling of plasma

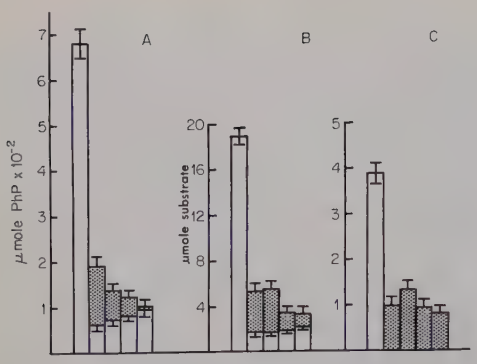


FIG. 10. Duodenal alkaline phosphatase (A) and jejunal maltase (B) and sucrase (C) in intact and hypophysectomized rats. In each group of five columns, the first column shows activity in intact rats at 24 days; the next four columns show activity in rats that were hypophysectomized at (left to right) 16, 14, 10, or 6 days. For the hypophysectomized groups the clear part of the column represents activity in normal animals at the age at which hypophysectomy was performed; the dotted area represents activity in operated animals at 24 days. Vertical lines indicate ±1 SEM. For more details, see Table 2.

insulin concentration that occurs between 15 and 25 days (Blasquez *et al.*, 1970) suggests a role for this hormone, which is known to selectively increase relative weight of the liver in hypophysectomized adult rats (Salter and Best, 1953) by promoting protein accretion (Cheek and Hill, 1970). The effect of hypophysectomy on insulin titer in sucklings is however unknown.

Since the intestine receives some of its own nutriment directly from the lumen (Levin, 1969; Levine *et al.*, 1974), the possibility exists that reduced food intake in hypophysectomized pups, possibly resulting from their hypothyroid state, might depress growth of the organ. Starvation decreases mucosal size in adults (Hoopes and Blair, 1958; Altmann, 1972); and conversely a large increase in size is associated with, if not caused by, hyperphagia in lactating females (Cairnie and Bentley, 1967). Deficient gastrin output resulting from diminished food intake might also be a contributing factor, since this hormone stimulates intestinal growth in unweaned pups (Lichtenberger and Johnson, 1974). The problem is not directly approachable by pair-feeding in nurslings. We did however attempt to evaluate the effect of reduced food intake by starving intact young rats on alternate days, beginning at 6 days. At 24 days these animals were not much heavier than those hypophysectomized at 6 days, but their small intestines were more than twice as heavy (Table 3). Hence underfeeding is probably not in itself a sufficient cause for the depressed growth of the intestine in hypophysectomized sucklings.

Despite the persistent small size of the intestine, which entails even a shortening of the villi to less than the 6-day height, differentiation of the epithelium does proceed to a limited extent in the hypophyseoprivic young. By 24 days the crypts deepen significantly, and the cells covering the villi assume a shape approximating that on the controls. The appearance of the apical cytoplasm and surface membrane suggests that closure is largely complete. The three enzymes we studied all change significantly in the 20–24-day period, achieving final levels in the range of those reached in

TABLE 3

BODY WEIGHT AND INTESTINAL WEIGHT AT 24 DAYS IN RATS ALLOWED TO NURSE CONTINUOUSLY (GROUP I), FED ON ALTERNATE DAYS (GROUP II), OR HYPOPHYSECTOMIZED AT 6 DAYS AND FED CONTINUOUSLY (GROUP III)

Group	Number	Body Weight (g)	Intestinal Weight (mg)	Intestinal Weight (mg/g body weight)
I	9	56.2 ± 2.3	2260 ± 121	38.5 ± 0.78
II	4	33.5 ± 1.8	1370 ± 93	40.7 ± 1.04
III	7	28.4 ± 1.9	631 ± 34	22.4 ± 0.74

he controls at 20 days. Because alkaline hosphatase, sucrase, and maltase are omponents of the surface membrane Johnson, 1967; Eichholz, 1969; Oda *et al.*, 969), their apparent activities are in part function of the amount of surface present er unit of intestinal length. The amount f mucosal surface per unit of serosal ength is greater in normal than in hypoph-sectomized rats at 24 days because the illi are taller in the former (and the micro-illi not consistently different in length or ensity); conversely, the slender columnar hape of the villi in the hypophysectomized nimals provides more villus surface per nit area than where the villi are nor-nally broad and leaflike. These opposing actors suggest that the amount of surface s not of great significance in determining evels of enzyme activity. The amount of ubmucosal tissue included in the total ho-nogenates we have used probably does not ontribute to the differences observed, ince the area external to the crypts is very hin (ca. 0.15 mm) in both experimentals nd controls at 24 days (Yeh and Moog, 974). In any case, the enzymatic differ-nces between normal and hypophyseo-rivic animals are more than merely quan-itative. Although duodenal phosphatase ctivity rises between 20 and 24 days in he absence of the pituitary, the tendency or activity with PhP to rise faster than hat with βGP is scarcely manifested, indi-ating that the isozymes peculiar to the dult murine duodenum (Etzler and Moog, 968: Moog and Yeh, 1973) have virtually ailed to appear. On the other hand, the nequivocal appearance of sucrase is a triking demonstration of differentiation, ince this enzyme is ordinarily not detecta-le as late as 16 days.

Evidently normal maturational proc-sses can begin in the absence of the hy-ophysis, and a timing mechanism contin-ues to regulate the onset of such processes. These conclusions are not altogether sur-rising, in view of the fact that the zona lomerulosa of the adrenal cortex is partly independent of the pituitary (Deane and Greep, 1946; Muller, 1971; Nussdorfer *et al.*, 1973). We have observed that the adrenals of hypophyseoprivic rat pups, though small, have broad, lipid-rich glomerular zones that probably secrete aldosterone. This hormone has glucocorticoid properties (Simpson and Tait, 1955) and is more effective than corticosterone in eliciting the appearance of sucrase in adrenalectomized sucklings (Koldovsky *et al.*, 1965). Thus aldosterone might substitute for missing products of the atrophied zona fasciculata in supporting the initial steps of intestinal differentiation. Whether or not an aldosterone-supported event could proceed to its culmination might be determined by the availability of thyroxine, which is no doubt deficient in hypophysectomized sucklings, in which the thyroid is small, with hypoplastic epithelium (Walker *et al.*, 1952; Yeh and Moog, unpublished observations). The importance of thyroxine is indicated by its effectiveness alone in reducing intestinal lactase to normal low levels in hypophysectomized rats (Yeh and Moog, 1974).

The fact that ablation of the pituitary at stages later than 6 days results in progressively less severe effects on enzyme accumulation indicates that the gland is active during the 2nd week, rather than suddenly intervening later to elicit an outflow of glucocorticoids. But even after operation at 6 days, there is a significant upsurge of phosphatase and disaccharidase activity between 20 and 24 days. What sets the time at which this and probably other correlated events occur remains a matter of conjecture. Maternal and dietary influences have been largely ruled out as determinants of the critical period of functional differentiation in the murine intestine (Moog, 1953, 1962), and our present findings apparently eliminate the hypothalamo-hypophyseal pathway as the primary mechanism determining when the culminating events in intestinal maturation shall begin.

To gain further insight into the role that glucocorticoids and other hormones play in the functional differentiation of the small intestine, it is obviously necessary to test the effects of replacement therapy in the hypophysectomized animals. Such studies are the subject of the following paper.

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Development of the Small Intestine in the Hypophysectomized Rat

II. Influence of Cortisone, Thyroxine, Growth Hormone, and Prolactin

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The intestinal deficiencies caused by hypophysectomy of rats at 6 days of age can be repaired to varying degrees by thyroxine or cortisone but not by growth hormone or prolactin. Administration of daily doses of thyroxine alone from 19–22 days raises duodenal alkaline phosphatase activity to normal levels at 24 days; it has a strong effect on jejunal sucrase and maltase, although these activities remain below those of controls. Thyroxine causes a marked increase in rough endoplasmic reticulum and restores the Golgi complexes to their normal appearance. It also elicits an intensification of periodic acid–Schiff (PAS) stainability of the brush border. Cortisone acetate given from 19 to 22 days elevates sucrase and maltase to normal levels but does not fully restore phosphatase activity. Like thyroxine, cortisone causes intensification of PAS staining of the brush border and also increases rough endoplasmic reticulum. It seems to stimulate Golgi activity, but results in the appearance of a variety of abnormal forms. The defects in Golgi configuration, brush border carbohydrate content, and activity of glycoprotein enzymes that are bound to the brush border may all reflect impaired glycosylation in the hypophyseoprivic state; the results of thyroxine or cortisone administration suggest that both hormones may affect glycosylation but in different ways.

INTRODUCTION

Removal of the pituitary gland from rats at 6 days of age causes severe deficiencies in intestinal development. Though structural maturation of the absorptive cells is not entirely prevented, the intestine is disproportionately small at the time when weaning normally occurs, the activities of enzymes characteristic of the post-sucking digestive–absorptive surface are subnormal, and the periodic acid–Schiff (PAS)-stainable substance fails to accumulate on the brush border (Yeh and Moog, 1975). Conversely, lactase activity remains at the high level appropriate to the neonatal stage (Yeh and Moog, 1974). These observations raise numerous questions about the hormonal control of intestinal maturation.

Considering the well-known influence of glucocorticoids on the functional differentiation of the small intestine (Moog, 1953; Clark, 1959; Doell and Kretchmer, 1964; Herbst and Koldovsky, 1972), the first question relates to the extent to which the effects of hypophyseoprivia result from lack of these hormones. Will exogenous glucocorticoid stimulate the activity of all enzymes, or will it act selectively? Since thyroxine has also been implicated in intestinal development, the second question concerns the need for this hormone. Will it act independently in the hypophyseoprivic state, or will it rather prove essential to provide support for the action of glucocorticoids? Taking into account the stunted growth of the intestine after pituitary ablation, the third question must be addressed to the relation between the enhanced growth and the differentiative events that normally take place concurrently as the intestine shifts from the infantile to the adult state. Is it necessary to stimulate growth in order to obtain normal biochemical differentiation? If so, will thyroxine and/or cortisone serve as growth-promoting agents, or is growth hormone itself required?

The purpose of the experimental studies described in this paper was to elucidate these and related problems by administering exogenous hormones to rats hypophy-

sectomized at 6 days of age.

MATERIALS AND METHODS

Rats of the NLR strain were used. Surgical methods as well as biochemical, histological, and ultrastructural techniques were the same as those already reported (Yeh and Moog, 1975). The following hormones were used: Cortisone acetate (Merck, Sharp and Dohme) suspension diluted in 0.9% saline; DL-thyroxine (Sigma), dissolved in 0.01 *M* NaOH; growth hormone (ovine, NIH-GH-S10 and -S11) in 0.9% saline; and prolactin (ovine, NIH-P-S10 and -S11) in 0.9% saline. Dosages, chosen to yield maximal enzyme activities, were 50 μg/g body weight/day for cortisone acetate, and 1 μg/g body weight/day for thyroxine. Growth hormone and prolactin were given at the rate of 20 or 500 μg/day. Controls were injected with equivalent amounts of saline or 0.01 *M* NaOH. Injections were intraperitoneal.

RESULTS

Influence of Cortisone and Thyroxine on Brush Border Enzymes

Duodenal alkaline phosphatase and jejunal α-disaccharidases normally reach maximal levels of activity in the middle of the 4th week. Taking into account the fact that it takes at least 72 hr for glucocorticoids to exert their full effects on enzyme levels (Moog, 1953; Doell and Kretchmer 1964; Herbst and Koldovsky, 1972), we de cided to test the effects of replacement ther apy in the 19–24-day interval. A singl dose of cortisone acetate administered t hypophysectomized animals at 21 days elic ited a very large increase of sucrase activ ity at 24 days but had lesser effects o maltase and alkaline phosphatase (Tabl 1). The same dose given daily from 19 to 2 or 22 days evoked greater augmentation c maltase and phosphatase activities, wit no difference between the effects of thre and four daily doses.

Administration of cortisone acetate fro 19 to 22 days elevated duodenal phospha tase activity at 24 days significantly abov that of untreated hypophysectomized rats as is also true when glucocorticoids ar given to intact sucklings, phenylpho phate (PhP)-hydrolyzing activity was a fected more than that with β-glycerpho phate (βGP), so that the PhP/βGP rati was also strongly elevated (Table 2). Th values attained were however below thos of the normal 24-day duodenum. Adminis tration of thyroid hormone from 19 to 2 days raised phosphatase activities to th control levels, and when both hormone were given, phosphatase activity with Ph rose to 186% of the level in intact animal (Table 2). Activity with βGP was affecte to a slightly lesser extent, so that th

TABLE 1

INFLUENCE OF A SINGLE INJECTION OF CORTISONE (C) ON ENZYME ACTIVITIES IN THE INTESTINES OF HYPOPHYSECTOMIZED RATS[a]

Group	Number	Duodenum		Jejunum		
		Protein (μg/mg)	Alkaline phosphatase (μmol of PhP)	Protein (μg/mg)	Maltase (μmol of substrate)	Sucrase
Hypex	5	150 ± 5.3	165 ± 21	149 ± 1.5	4.12 ± 1.15	0.72 ± 0.21
			$P > 0.10$		$P < 0.025$	$P < 0.01$
Hypex + C	6	157 ± 7.7	224 ± 40	152 ± 5.7	8.03 ± 0.58	2.46 ± 0.42
			$P < 0.001$		$P < 0.001$	$P > 0.1$
Intact	6	155 ± 5.3	616 ± 31	149 ± 2.1	17.10 ± 1.08	3.37 ± 0.45

[a] Rats were hypophysectomized at 6 days, given cortisone acetate (50 μg/g body weight) at 21 days sacrificed at 24 days. Activities (± SEM) are per milligram of protein per hour. Significance of difference between each value and the one below determined by Student's *t* test.

TABLE 2

INFLUENCE OF CORTISONE (C) AND THYROXINE (T) ON ENZYME ACTIVITIES IN THE INTESTINE OF HYPOPHYSECTOMIZED RATS[a]

Group	Number	Duodenal phosphatase			Jejunal disaccharidase	
		PhP (μmol of substrate)	βGP	PhP/βGP	Maltase (μmol of substrate/mg of protein)	Sucrase
Intact (I)	9	655 ± 43	185 ± 5.4	3.54 ± 0.14	18.70 ± 1.16	3.33 ± 0.21
Hypex	7	102 ± 16	103 ± 12	0.98 ± 0.06	4.41 ± 0.86	0.88 ± 0.20
Hypex + C	9	437 ± 67	179 ± 22	2.35 ± 0.21	15.33 ± 1.64	2.93 ± 0.08
Hypex + T	9	675 ± 52	212 ± 12	3.39 ± 0.18	10.55 ± 0.93	1.70 ± 0.12
Hypex + C + T	6	1216 ± 106	308 ± 27	3.79 ± 0.08	18.82 ± 1.36	3.30 ± 0.63
P values						
HC vs I		<0.025	0.50		>0.10	<0.05
HT vs I		0.50	<0.05		<0.001	<0.001
HC vs Ht		<0.025	>0.10		<0.025	<0.001
I vs HCT		<0.001	<0.005		0.50	0.50
HC vs HCT		<0.001	<0.005		>0.10	0.50
HT vs HCT		<0.001	<0.005		<0.001	<0.050

[a] Cortisone acetate (50 μg/mg body weight/day) or thyroxine (1 μg/g/day) were injected from 19 to 22 days. See also footnote to Table 1.

PhP/βBP ratios were increased above the normal value.

Responses of the α-disaccharidases in the jejunum to hormone dosage from 19 to 22 days were rather different from that of phosphatase. When cortisone was given, maltase and sucrase activities were below those of intact 24-day animals but not significantly so (Table 2). On the other hand, thyroxine was much less effective in elevating these enzymes (Table 2). When both hormones were administered, maltase and sucrase activities rose to the level of the intact controls but not above it. These results are in contrast to those previously obtained with jejunal lactase, which can be reduced to the 24-day level in hypophysectomized animals by thyroxine but not by cortisone (Yeh and Moog, 1974).

Influence of Cortisone and Thyroxine on Body and Intestinal Weight

Body growth is not favorably affected by injection of cortisone or thyroxine according to the regimen that normalized intestinal enzyme activities. On the contrary, both hormones further reduce the retarded growth of the hypophysectomized animals (Table 3). The growth of the small intestine is less affected, with the result that the relative weight or the organ is increased slightly over that in untreated experimentals, though remaining far below that of intact animals at 24 days. The effect of thyroxine is more severe than that of cortisone; and, when the two hormones are administered in concert, cortisone appears to provide some protection against the wasting effects of the relatively large amount of thyroxine used in these experiments.

The lack of influence of cortisone and thyroxine on intestinal weight is corroborated by study of the size of villi and crypts. Neither hormone alone significantly altered the height of the villi or the depth of the crypts in hypophyseoprivic rats (Table 4). Thyroxine alone raised the mitotic index by 16.6%. When the hormones were given in concert, small but significant increases were elicited in villus and crypt size, and mitotic index was the same as with thyroxine alone. When viewed in the scanning electron microscope, the villi of cortisone- or thyroxine-treated pups were found to retain the narrow tongue-like

TABLE 3

INFLUENCE OF CORTISONE (C) AND THYROXINE (T) ON BODY WEIGHT AND INTESTINAL WEIGHT OF HYPOPHYSECTOMIZED RATS[a]

Group	Number	Body weight		Small intestine		
		(g)	(*P*)	(mg)	(*P*)	(mg/g body weight)
Intact	8	62.4 ± 2.4		2260 ± 54		36.6 ± 1.2
Hypex	8	33.8 ± 0.9		783 ± 30		23.2 ± 0.6
Hypex + C	8	29.3 ± 1.2	<0.01	742 ± 30	>0.2	25.4 ± 0.7
Hypex + T	7	27.6 ± 2.0	<0.025	678 ± 52	<0.05	24.6 ± 0.7
Hypex + C + T	6	30.3 ± 0.8	<0.025	811 ± 58	>0.5	26.9 ± 2.2

[a] Rats were hypophysectomized at 6 days, injected with hormones from 19 to 22 days, sacrificed at 24 days. *P* values refer to difference between treated groups and untreated hypophysectomized group. See footnotes to Tables 1 and 2.

TABLE 4

INFLUENCE OF CORTISONE (C), THYROXINE (T), AND GROWTH HORMONE (GH), ON INTESTINAL DIMENSIONS AND MITOTIC INDEX[a]

Group	Number	Height of villi (number of cells)	Depth of crypts (number of cells)	Mitotic index (%)
Intact	7	78.5 ± 2.3	19.1 ± 0.37	4.05 ± 0.13
Hypex	7	51.7 ± 2.0	11.3 ± 0.60	2.70 ± 0.06
Hypex + C	5	54.5 ± 1.7	12.8 ± 0.49	2.71 ± 0.13
Hypex + T	5	51.6 ± 0.6	12.9 ± 0.41	3.15 ± 0.09
Hypex + C + T	4	59.8 ± 3.2	15.1 ± 0.74	3.11 ± 0.11
Hypex + GH	5	53.8 ± 1.6	11.6 ± 0.50	2.60 ± 0.18
Hypex vs HT: *P*				<0.025
Hypex vs HCT: *P*		<0.05	<0.005	<0.01

[a] Hypophysectomized at 6 days, given cortisone and/or thyroxine from 19 to 22 days or growth hormone (500 μg/day) from 16 to 23 days, sacrificed at 24 days. For methods of obtaining data, see Table 1 in Yeh and Moog (1975). $P > 0.05$ for all differences between treated and untreated hypophysectomized rats except where specified.

shape seen in untreated specimens at 14 days (Yeh and Moog, 1975).

Influence of Cortisone and Thyroxine on Cell Structure

In rats hypophysectomized at 6 days, the absorptive cells of the villous epithelium attain partially normal structural differentiation at 24 days (Yeh and Moog, 1975). The most striking differences brought about by the administration of cortisone or thyroxine to the operated animals affect the distribution of ribosomes and the character of the Golgi zone. After treatment with either hormone, a high proportion of ribosomes are associated with endoplasmic reticulum membrane, with rough-surfaced cisternae being predominantly aligned along the surfaces of mitochondria. Whether the total number of ribosomes in a cell is increased by hormone treatment cannot be decided from the pictures. Both hormones also affect Golgi structure but in different ways.

The Golgi membranes in normal 24-day-old rats occur as prominent elongated stacks that are usually vesiculated along both faces (Fig. 1). In hypophysectomized animals, however, the membranes are short; they are often associated with large vacuoles containing granular material, but sometimes only short lengths of densely packed membranes are seen (Fig. 2). Treatment with thyroxine restores the

Golgi complexes to their normal configuration (Fig. 3). By contrast, cortisone leads to gross disorganization of Golgi structure. Abnormally large vacuoles are commonly found in the supranuclear zone. These vacuoles are sometimes associated with smooth membranes that may represent distorted Golgi sacs (Fig. 4A); frequently the vacuoles include or are bounded by granular membranes (Figs. 4A and B). Stacks of granular membranes also appear, in conjunction with vesicles that are abnormal in size and placement (Figs. 4C and D); usually such stacks are quite short.

Mitochondria are long, slender, and often branched after treatment with either hormone, as they are in intact or untreated hypophysectomized animals (Yeh and Moog, 1975). Thyroxine has no special effect on mitochondrial appearance (Fig. 3).

The microvilli, which are indistinguishable in hyphyseoprivic animals and controls, are not affected by hormone administration. Application of the PAS stain for polysaccharides, however, reveals a marked difference in the stainability of the brush border. Normally there is a marked intensification of staining between 6 and 24 days (Figs. 5A and B). In hypophysectomized pups at 24 days, however, the stain is very weak, but thyroxine and cortisone, administered between 19 and 22 days, evoke a striking increase in the staining reaction at 24 days, with the effects of the two hormones combined being apparently additive (Figs. 5C–F).

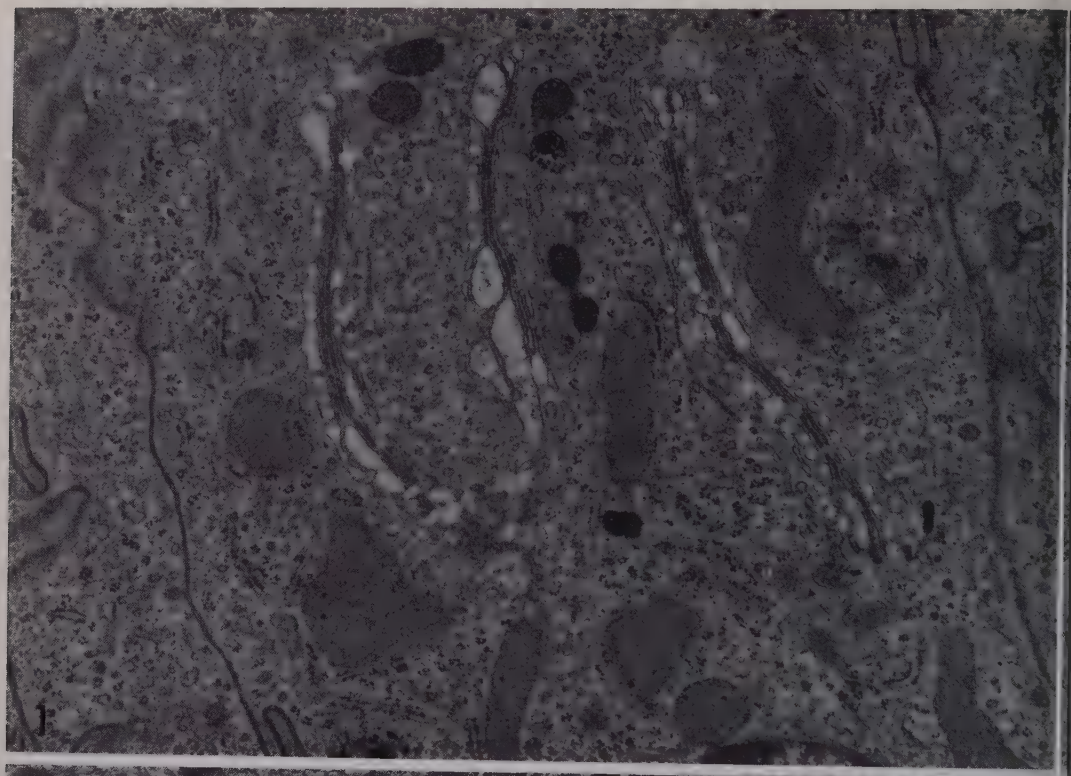
Influence of Growth Hormone and Prolactin

Hypophysectomized sucklings were given growth hormone (20 or 500 μg/day) from 16 to 23 days, this 8-day schedule being chosen because it approximates a standard assay method for growth hormone in young adult rats (Parlow *et al.*, 1965). Prolactin (500 μg/day) was also given from 16 to 23 days. Neither hormone exerted any effect on body weight or intestinal weight. Growth hormone did not affect mitotic index or size of villi and crypts (Table 4). It had no effect on duodenal phosphatase activity assayed with either PhP or βGP and no effect on jejunal maltase, but it depressed sucrase activity slightly (Table 5). Lactase, which is decreased by cortisone or thyroxine (Yeh and Moog, 1974), was unaffected. Prolactin also failed to alter these activities.

DISCUSSION

The results reported in this paper are partly expected, partly unexpected. That intestinal enzyme deficiencies caused by hypophyseoprivia should be largely repairable by administration of glucocorticoids is in agreement with a large body of data showing that the increases of alkaline phosphatase, maltase, and sucrase occurring during normal development are dependent on the adrenal cortex (for references, see Yeh and Moog (1975)). That cortisone should fail to elevate phosphatase activity to its normal level in hypophysectomized rat pups is also in accord with evidence that thyroid status influences the efficacy of the adrenal hormone (Moog, 1961; Watson and Tuckerman, 1971). That thyroxine alone should exert strongly positive effects in the face of severe glucocorticoid deficiency does however reveal that the thyroid gland must play a more than merely supportive role in intestinal maturation. In studies now being prepared for publication, we have found that the dosage of thyroxine that exerts strong effects on intestinal enzymes does not elevate the plasma corticosterone titer above the low levels found in intact sucklings up to 18 days of age (Daniels *et al.*, 1973).

The striking effects of hypophysectomy and hormone therapy on the morphology of Golgi may be related to the role of this organelle in the synthesis of glycoproteins or lipoproteins or both. Golgi membranes are implicated in the production of enveloping membranes for chylomicrons (Friedman and Cardell, 1972). If hypophysec-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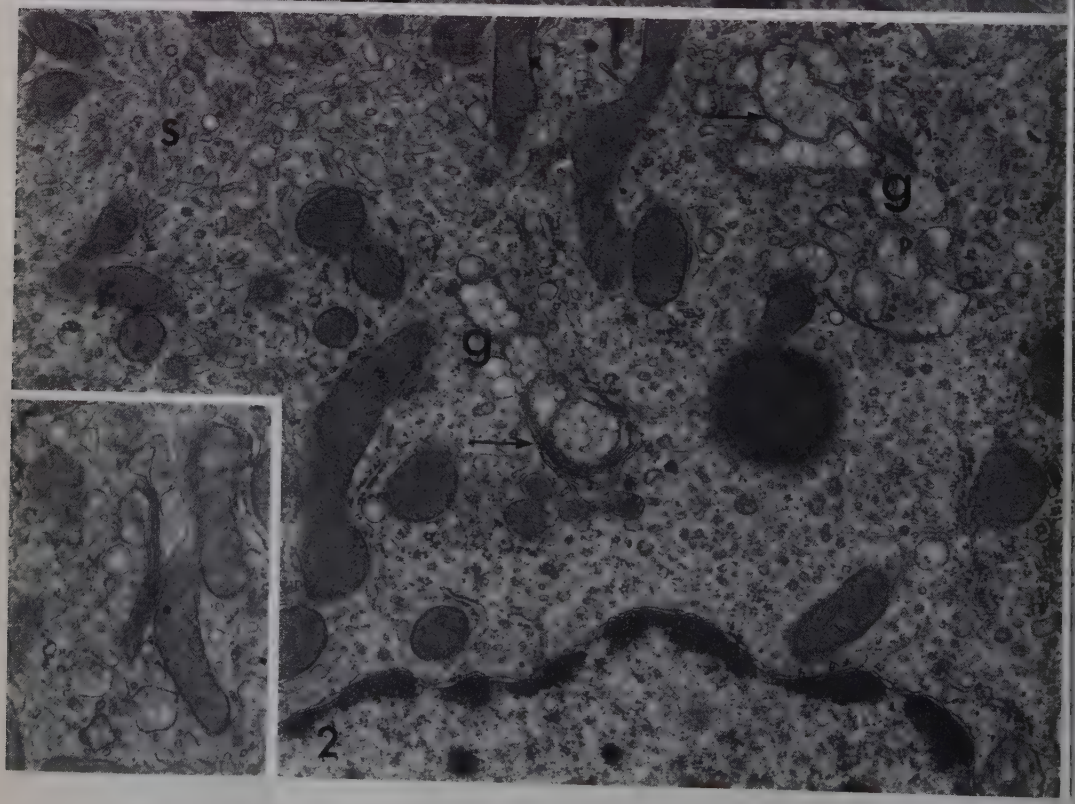
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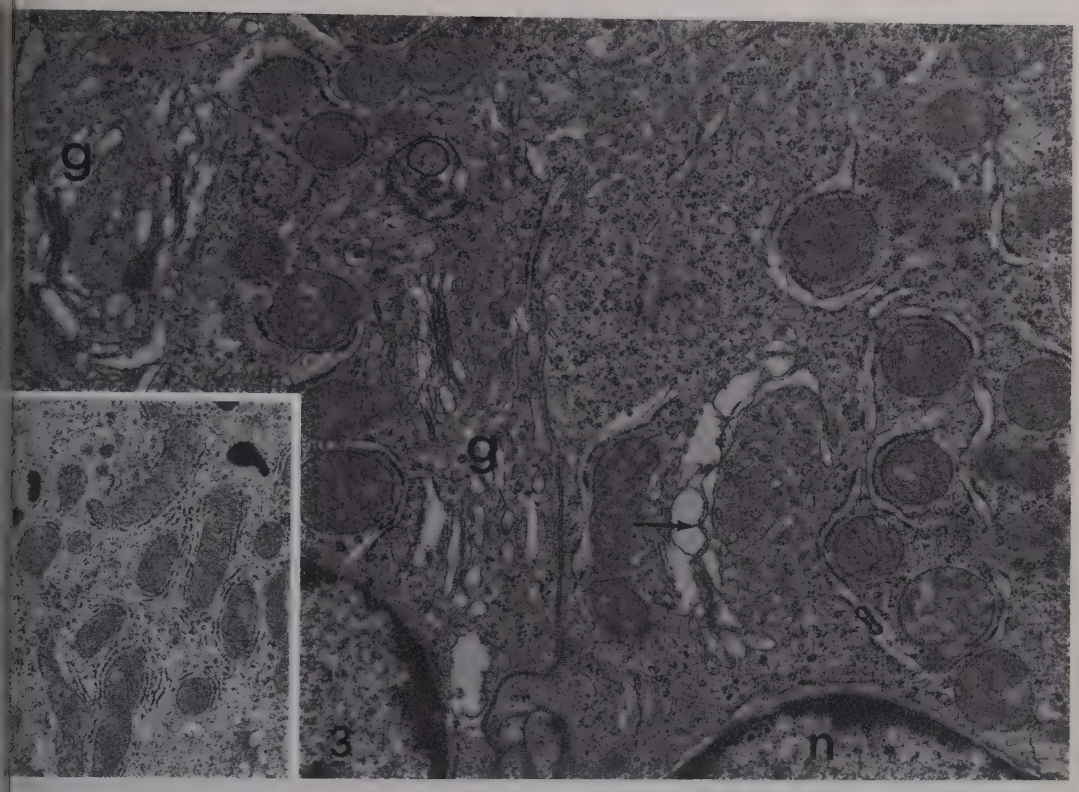

FIG. 3. Rats hypophysectomized at 6 days, given thyroxine at 19–22 days, sacrificed at 24 days. Cells halfway up villus. The cell at the left has several active Golgi complexes (g); the one at the right has a single Golgi sac (arrow) associated with large vacuoles. Nucleus (n) is at lower right. Free ribosomes are abundant and the mitochondria are surrounded by expanded cisternae of rough endoplasmic reticulum. 19,500×. Inset: More apical region of cell, showing normal form of mitochondria. 9100×.

tomy brings about enhanced incorporation of fatty acids into esters in nurslings, as it does in adults (Gelb, 1966), without concomitant stimulation of chylomicron envelop formation, the vacuolization of the Golgi zone illustrated in Fig. 2 might be in part explained, although the fact that the increased incorporation rate of fatty acids is reversed to an equal degree by either cortisone or thyroxine is in contrast to the very different morphological effects of these hormones in our studies. In rabbit liver, however, large clusters of very low density lipoprotein (VLDL) particles appear in association with Golgi after 4 days of cortisone treatment (Mahley *et al.*, 1968); the vesiculation we have observed following administration of cortisone might be due to hyperactive production of VLDL, which is synthesized by intestinal mucosa as well as by hepatocytes (Mahler *et al.*, 1971).

The increased association of ribosomes with endoplasmic reticulum membrane

FIGS. 1 and 2. Electron micrographs showing the supranuclear zone of absorptive cells halfway up the villus in 24-day-old rats. Fig. 1, Intact rat. Three stacks of Golgi membranes are shown. Note approximately equal vesiculation on both faces of the two lateral stacks. The dense granular bodies may be products of Golgi activity. Large number of free ribosomes are evident; rough endoplasmic reticulum was more abundant apical to the region shown. Nucleus is at bottom right. Fig. 2, Rat hypophysectomized at 6 days. Golgi membranes are short (arrow at left) or occur as single sacs (arrows at right). Attendant vesicles are filled with amorphous material. Smooth endoplasmic reticulum(s) is abundant, but there is little rough ER. Inset: A stack of compact Golgi sacs. All 19,500×.

TABLE 5

INFLUENCE OF GROWTH HORMONE (GH) AND PROLACTIN (P) ON SUCRASE ACTIVITY IN HYPOPHYSECTOMIZED RATS[a]

Group	Dose (μg/day)	Number	Sucrase (μmol)	(P)
Hypex		7	0.72 ± 0.08	
Hypex + GH	500	5	0.33 ± 0.10	<0.025
Hypex + GH	20	5	0.45 ± 0.08	<0.05
Hypex + P	500	6	0.50 ± 0.09	>0.10

[a] Hypophysectomized at 6 days, given hormone from 16 to 23 days, sacrificed at 24 days. *P* values refer to difference between untreated group and each treated group.

that occurs in normal development or in response to thyroxine or cortisone administration is compatible with enhanced synthesis of both intracellular and extracellular proteins (Campbell, 1970). Although this problem has not been specifically studied in the intestinal epithelium, there is little reason to doubt that much of the proliferation of membrane capable of linking with ribosomes is required for the synthesis of the abundant glycoproteins that are transported to the surface of the cells, where they comprise the compartment from which absorption occurs (Parsons and Boyd, 1972; Ugolev, 1972). This view links the increase or rough endoplasmic reticulum with the concomitant age- or hormone-dependent events we have observed, viz., the prominence of the Golgi complex, the amount of PAS-stainable substance on the brush border, and the activity of enzymes of the surface membrane.

There is ample evidence that newly synthesized proteins that are to be passed through the plasma membrane move from rough to smooth endoplasmic reticulum and thence into Golgi cisternae (Jamieson and Palade, 1967; Beams and Kessel, 1968; Campbell, 1970), where they are glycosylated before being released in vesicles in which they may move to the cell surface (Rambourg, 1971; Bennett *et al.*, 1974). In an elegant series of studies of the absorptive cells of the intestinal epithelium, Leblong and his co-workers have demonstrated that tritiated saccharides are swiftly taken up by the Golgi complexes, subsequently appearing in plasma membranes, with the greatest accumulation in the brush border (Neutra and Leblond, 1966; Bennett, 1970; Bennett and Leblond, 1970). Hence the atypical configuration of the Golgi membranes in hypophysectomized animals may be causally related to the deficient PAS-stainability of the epithelial surface.

The lack of stainable carbohydrate at the surface may in turn be related to the failure of enzyme activities to rise normally. Alkaline phosphatase, maltase, and sucrase, which are components of the surface membrane (Miller and Crane, 1961; Eichholz, 1969), are glycoproteins (Forstner, 1970; Saini and Done, 1970; Kelly and Alpers, 1973). When newly glycosylated proteins are extracted from the microvillus plasma membrane, they are found to include the hydrolytic enzymes of the membrane (Forstner, 1971); and the degree of incorporation of labeled monosaccharides, like the distribution of alkaline phosphatase and disaccharidase, rises from villus base to tip (Weiser, 1973). Both histochemical (Hugon and Borgers, 1966) and immunochemical (Lietz *et al.*, 1974) evidence has demonstrated the presence of alkaline phosphatase in the Golgi zone, where it is presumably receives its saccharide components. Although disaccharidases have not yet been directly linked to Golgi, it is known that they are synthesized intracellularly in active form (Grand *et al.*, 1972).

Though the proliferation of rough endoplasmic reticulum membrane may be provisionally regarded as a common step by which both glucocorticoids and thyroxine promote the differentiation of the intestinal epithelium in hypophysectomized sucklings, each hormone must also exert individual effects. The conspicuous difference in their effect on Golgi structure might be due to a stronger influence of

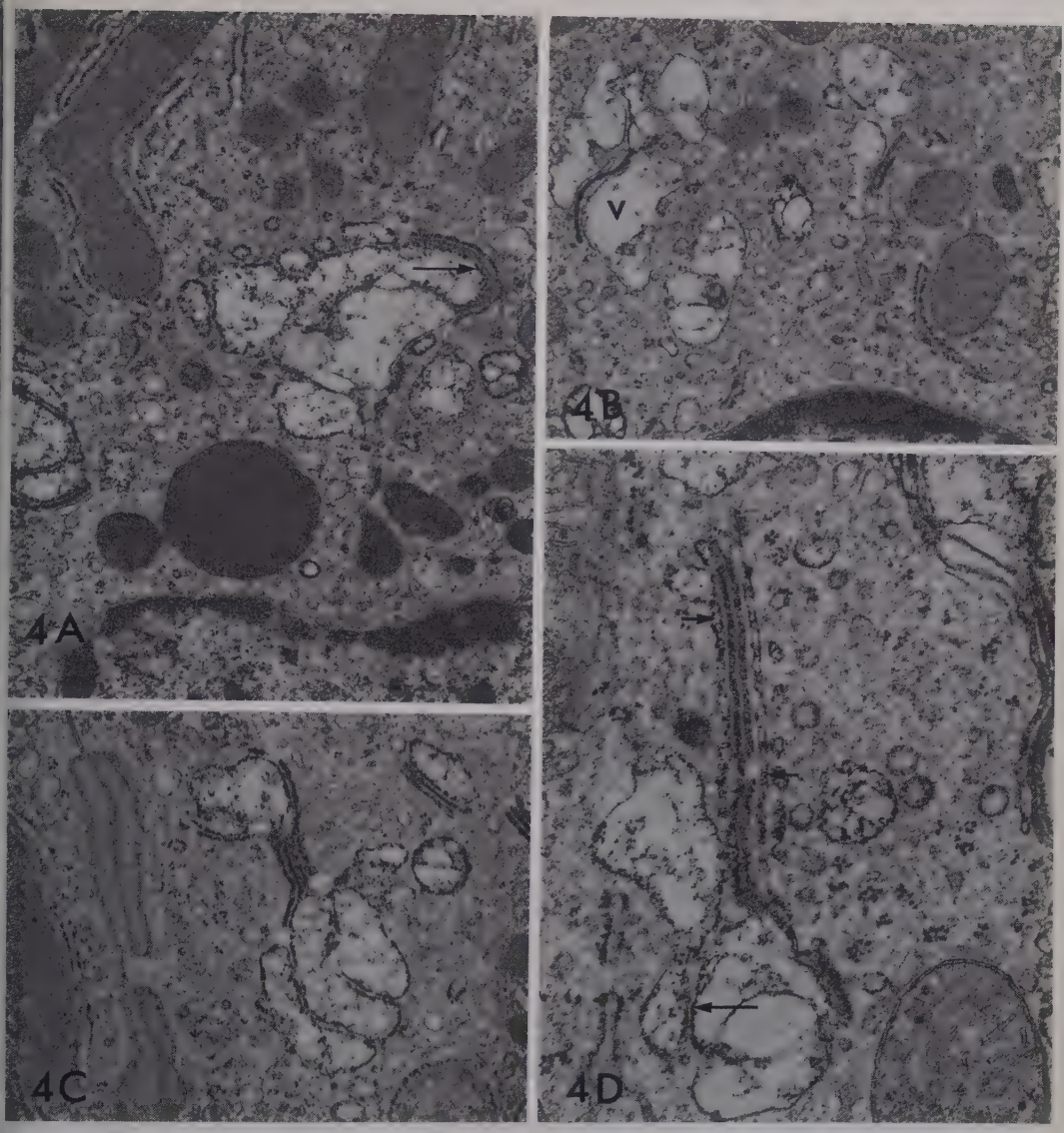

FIG. 4. Rats hypophysectomized at 6 days, given cortisone at 19–22 days, sacrificed at 24 days. Cells halfway up villus. (A), Probable Golgi membranes (arrows) associated with granules and very large vacuoles. Typical rough endoplasmic reticulum is abundant. (B), Large supranuclear vacuoles (v) at the left surrounding a granular membrane. (C), A short stack of granular membranes terminating in large vacuoles with much included matter. The neighboring cell contained several complexes of this type. All 19,500×. (D), Granules are seen to be enclosed within Golgi sacs (short arrows) and appear on the inner surfaces of associated vacuoles (long arrow). 34,500×.

cortisone of VLDL synthesis, as pointed out before. Another striking difference is the lesser effectiveness of thyroxine in raising maltase and sucrase to the levels found in intact animals. Since increase in activity of these two enzymes involves *de novo* synthesis (Doell *et al.*, 1965; Galand and Forstner, 1974), our results may indicate that cortisone is more effective in stimulating synthesis of the protein components of these enzymes. Even if this is true, however, it has not been demonstrated that disaccharidase activity has been completely normalized by cortisone. In view of

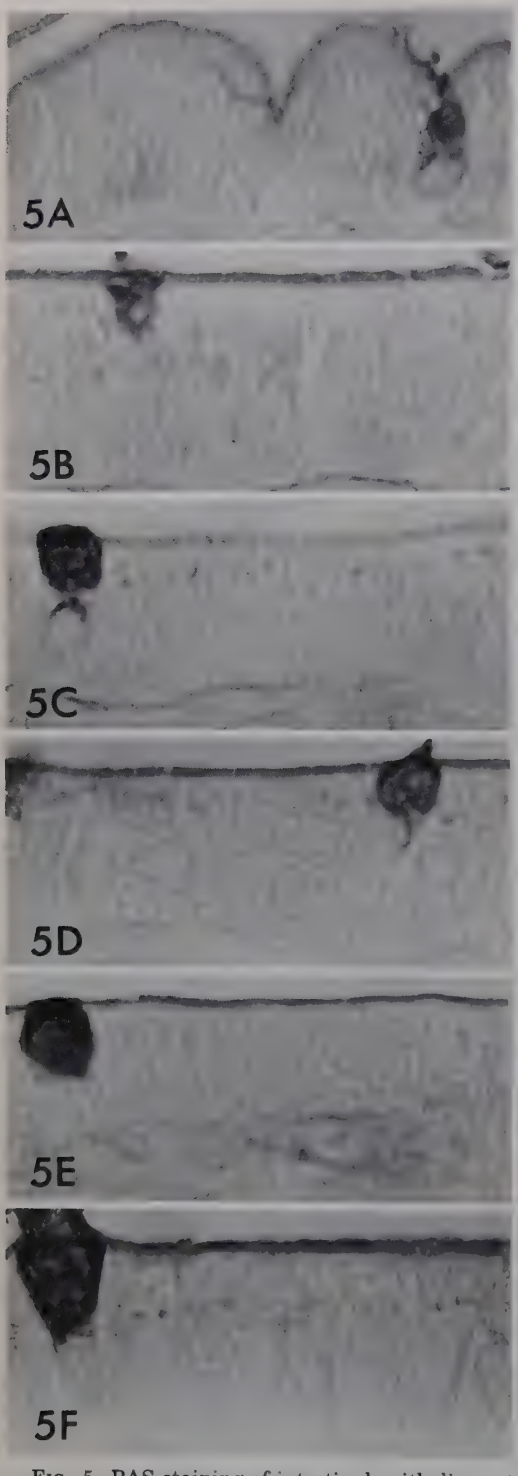

FIG. 5. PAS staining of intestinal epithelium at 6 days (A) and 24 days (B–F). (A), Brush border has lightly stained inner layer and unstained outer layer. (B), Intact animal, brush border is deeply stained. (C), Hypophysectomized at 6 days, brush border is pale with unstained outer layer. (D–F), Hypophysectomized at 6 days, given cortisone (D), thyroxine (E), or cortisone + thyroxine (F) at 19–22 days; border is deeply stained. One goblet cell included in each strip. 1150×.

the hypothesis that glycosylation of proteins provides "informational" factors that enable the proteins to be properly localized (Whaley *et al.*, 1972; Winterburn and Phelps, 1972), it is an interesting possibility that the maltase and sucrase processed in the cortisone-disordered Golgi saccules do not become fully invested in the surface membrane but remain in part within the cell (Grand *et al.*, 1972).

By contrast with the disaccharidases, alkaline phosphatase in hypophysectomized pups is elevated to the control level by thyroxine but not by cortisone. The phosphatase in question is the type, peculiar to the adult murine duodenum, that is characterized by high activity on PhP relative to βGP (Moog, 1961; Moog and Yeh, 1973) and that comprises a distinct series of isozymes apparently graded in PhP/βGP activity from villus base to tip (Moog *et al.*, 1969). It is preceded by an enzymatically inactive form (Etzler and Moog, 1966), a fact which supports the suggestion that its appearance may involve activation rather than *de novo* synthesis primarily. If the activation process entails serial change in the carbohydrate components, the ability of thyroxine to raise phosphatase activity and PhP/βGP ratio to the normal level in hypophysectomized pups might be explicable in terms of the observed rectification of Golgi structure by this hormone. It may be pertinent that cortisone and thyroxine together elevate phosphatase (but not disaccharidase) to supranormal levels. If this result is due to stimulation of an activation mechanism that involves glycosylation, it may be in turn related to the additive effect of the two hormones on the amount of PAS-stainable substance in the duodenal brush border.

In intact animals the developing tissues

are affected first by heightened levels of thyroxine, which reaches its peak concentration in the plasma between 14 and 16 days (Samel, 1968; Clos *et al.*, 1974), before corticosterone titer has begun its climb to adult levels (Daniels *et al.*, 1973; Yeh and Moog, unpublished). Possibly the failure of the glucocorticoid to elicit normal differentiation reflects the need for prior conditioning by thyroxine. In addition, the early increase of the thyroid hormone may account for the progressively less severe effects of hypophysectomy on intestinal development when the operation is performed later than 6 days (Yeh and Moog, 1975).

The failure of growth hormone and prolactin to enhance growth in the hypophysectomized animals leaves open the question of what controls growth in the nursling stage (see Yeh and Moog, 1975). In thyroidectomized pups, 0.25 μg of thyroxine per day restores normal growth during the first 3 weeks (Clos *et al.*, 1974), and 2.5 μg per day is effective after hypophysectomy at 21–23 days (Scow, 1954). The doses of thyroxine that we have used were apparently large enough to inhibit rather than promote growth. But this fact makes it more striking that intestinal differentiation can be strongly stimulated by developmental hormones without any previous or concurrent effect on growth. It appears that the spurt in intestinal growth that normally occurs in the 3rd week (Herbst and Sunshine, 1969; Yeh and Moog, 1975) is not essential to the differentiative processes that go on concurrently.

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An Autoradiographic Study of the Origin of Intestinal Blastemal Cells in the Newt, *Notophthalmus viridescens*[1]

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Fifty adult newts were used in this investigation; in 44 animals, the intestine was transected perpendicular to its longitudinal axis approximately midway between pylorus and rectum. The free ends of the intestine were held in apposition with a single suture and replaced into the coelom. The animals were injected intraperitoneally with [^{3}H]thymidine from 0 to 35 days after transection of the intestine and killed 6 hr later. In nontransected, control intestines, the only tissue that incorporated [^{3}H]thymidine was the mucosal epithelium. In transected intestines, only the mucosal epithelium labeled in animals which had been injected with [^{3}H]thymidine from 0 to 4 days after the intestine was incised. Later on, serosal cells and smooth muscle cells of the intestinal stump underwent morphological alteration, initiated the incorporation of [^{3}H]thymidine into DNA, and began replication. At 6 days after transection, serosal cells adjacent to the plane of transection were incorporating [^{3}H]thymidine and, at 12 days, smooth muscle cells at the transected surface were labeling. It seems probable that they both furnished cells to the intestinal blastema; the lining epithelium of the mucosa, however, did not appear to contribute to the blastema proper.

INTRODUCTION

Regeneration in amphibians has been studied since the middle of the 18th century when Spallanzani (1768) demonstrated that newts could regenerate functional limbs. Over a century later, Colucci (1891) learned that salamanders regenerated a new lens upon removal of the old one. It was not until the 1950's, however, that the intestines of frogs (Goodchild, 1954a, b, 1955, 1956; O'Steen, 1959b) and salamanders (O'Steen, 1958, 1959a) were found to regenerate.

Upon transection of the intestine of adult *Rana pipiens,* Goodchild (1956) observed that blastemas formed on the cut ends. The early blastema was composed primarily of fibroblasts in a meshwork of collagenous fibers but agranulocytes, granulocytes, and mesothelial cells were also present within it. Smooth muscle cells often became isolated as islands in the blastema and transformed from spindle-shaped cells with elongate, compact nuclei into ovoid cells with spherical, vesicular nuclei. According to Goodchild, mucosal epithelial cells dedifferentiated in the blastema and new mucosal epithelium appeared to arise either *de novo* from cords of small cells or by migration of the existing epithelium. The blastema appeared to receive cellular contributions from all local intestinal tissues, i.e., serosa, smooth muscle, submucosal connective tissue, and mucosal epithelium. In *Rana clamitans* tadpoles, however, O'Steen (1959b) found that the blastema formed from cells of the serosa, muscularis externa, and submucosa but not from mucosal epithelial cells.

In adult *Notophthalmus viridescens,* O' Steen (1958) observed that, upon complete transection of the intestine, the mucosa rolled out and contacted the serosa on the outside of the intestine adjacent to the plane of amputation. Subsequently the serosa folded over on itself while the submucosa and muscularis externa rolled around

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the transected ends of the intestine. The submucosa then became edematous and adhesions occurred between the everted tissues and the serosa. Serosal cells, immediately adjacent to the cut ends, enlarged and became spherical to cuboidal in shape; each had a large, globular nucleus. Smooth muscle fibers in the muscularis externa dissociated and began to dedifferentiate. Meanwhile, the mucosal epithelium proliferated, migrated, and formed epithelial extensions in the area of the everted tissues. Then, a blastema, composed of fibrocytes and collagenous fibers, developed at the cut surfaces of the intestine with isolated groups of mucosal epithelial cells interspersed among the collagenous fibers. The author believed that some of these epithelial cells subsequently dedifferentiated and became part of the blastema. In addition, cells from the serosa, smooth muscle, and submucosa also appeared to contribute to the blastema.

In subsequent studies of intestinal regeneration in the newt (O'Steen, 1961; O'Steen and Walker, 1962), autoradiography was used in an attempt to determine which cells of the intestine were giving rise to blastemal cells. In order to label mucosal epithelial cells exclusively (O'Steen and Walker, 1960, 1962), [^{3}H]thymidine was injected into animals 10 days prior to intestinal transection at a time when mitotic activity was confined to these cells. By fixing at successive intervals after transection, the epithelial cells could be followed during the regenerative process. It was found that the primary role of the mucosal epithelium in regeneration was one of growing into the blastema, migrating through it, and fusing with a similar outgrowth from the other end.

As in intestinal regeneration, limb regeneration in salamanders involves formation of a blastema at the distal end of the remaining stump. The most widely accepted source of limb blastemal cells is the local tissues of the limb stump, except the epidermis (reviews by Chalkley, 1959; Goss, 1961; Hay, 1962, 1966; Thornton, 1968). This was perhaps most definitively shown by autoradiographic studies of limb regeneration, using intraperitoneal injection of [^{3}H]thymidine, in which skeletal muscle, cartilage, and perichondrial cells (O'Steen and Walker, 1961) or cells of skeletal muscle, loose connective tissue, endomysium, epimysium, nerve sheaths, and periosteum (Hay and Fischman, 1960, 1961) became labeled and contributed to the regeneration blastema.

Therefore, because of the important information obtained from these autoradiographic studies on the origin of limb blastemal cells, it was thought that more specific information about the origin of intestinal blastemal cells could be gained if similar experiments were performed on the regenerating intestine. The present paper reports the results of such studies.

MATERIALS AND METHODS

Common newts, *Notophthalmus (Diemictylus, Triturus) viridescens viridescens* (Rafinesque), collected in Massachusetts or from ponds in the vicinity of Morgantown, W. Va., were used throughout the experiment. They were maintained at room temperature (20–25°C) in aquaria containing dechlorinated tap water and fed chopped beef liver three times weekly.

[^{3}H]thymidine (Schwarz/Mann), designated thymidine[methyl-^{3}H], with a specific activity of 16 Ci/mmol or 23.5 Ci/mmol and a concentration of 0.5 mCi/ml was used throughout the investigation. The isotope was diluted with sterile distilled water to 100 μCi/ml and injected at 5 μCi/g body weight intraperitoneally at a point away from the lateral body wall incision in order to avoid loss of isotope through an incompletely healed wound. To avoid possible diurnal cyclic variation in isotope utilization, all injections were made between 0800 and 1000 hr.

All operations were performed under a dissecting microscope with iridectomy scissors and watchmaker's forceps. The ani-

mals were completely submerged during the operation in Holtfreter's solution contained in deep petri dishes. Tris–HCl was used instead of sodium bicarbonate to buffer this solution to a pH of 7.0–7.1 (Reyer, 1962). Prior to operation, animals were starved at least 1 week to decrease the amount of material in their intestinal tracts and, just before operating, were anesthetized in 0.1% Chloretone (Parke, Davis) in Holtfreter's solution for 20–40 min. The method of operation was similar to that of O'Steen (1958). An incision of approximately 1 cm was made just ventral to the line of dorsal pigmentation in the caudal one-third of the right lateral body wall. A loop of intestine was carefully pulled through the incision and the intestine transected perpendicular to its longitudinal axis approximately midway between pylorus and rectum. The transection usually involved a small portion of dorsal mesentery and a single, loose, black silk suture was placed through the two intestinal stumps on the side away from the dorsal mesentery to hold the free ends in approximate apposition. The body wall incision was sutured and animals were allowed to recover in moist chambers at 12–15°C for 12–14 hr followed by room temperature for 1–2 weeks. Subsequently they were placed in dechlorinated tap water in individual 1-quart glass bowls.

[^{3}H]thymidine was injected into animals immediately upon transection of the intestine, at 4 and 12 hr posttransection, and at 1, 2, 4, 6, 8, 10, 12, 15, 20, 25, 30, and 35 days after transection. Six hours after injection, a short piece of intestine, including the blastema and adjacent transected area, was removed for fixation. For controls, two well-fed and two starved normal animals as well as sham operated[3] animals were similarly injected with [^{3}H]thymidine and a piece of intestine removed and fixed 6 hr later.

Tissues were fixed either at room temperature in Bouin's fluid or at 2–4°C or room temperature in Carnoy's A fluid (Lillie, 1965) for 18–24 hr, dehydrated in ethanol, cleared in terpineol for 1–2 weeks, rinsed in benzene for 0.5 hr, and embedded in paraffin (Paraplast, Fisher). Seven-micrometer serial sections were cut and mounted on chemically clean glass slides with a gelatin–water solution (50 mg of Eastman purified, calfskin gelatin/25 ml).

Autoradiographs were prepared according to the methods of Hay and Fischman (1961), Kopriwa and Leblond (1962), and Rose and Rose (1965) as summarized in Reyer (1971). The sections were deparaffinized in xylene, treated with absolute alcohol, absolute alcohol plus absolute ether (1:1), and pure absolute ether for 5 min each, and air dried for 1 hr. The slides were dipped in melted Kodak NTB3 nuclear track emulsion, dried at 28°C and 80% relative humidity, and exposed at 3–4°C for 1–4 weeks. They were developed in Kodak D-11 at 14°C for 4 min and stained through the emulsion with Ehrlich's acid hematoxylin and erythrosin or nuclear fast red and indigo–carmine (Mortreuil-Langlois, 1962).

RESULTS

The [^{3}H]thymidine-labeling pattern in normal, nontransected intestines was the same in animals that had been fed and in those that had been starved for 3 weeks prior to injection of the isotope. Labeled cells were present primarily in cell-nests (Patten, 1960) of the mucosal epithelium but occasionally in the surface epithelium also. Connective tissue cells of the submucosa or lamina propria were very infrequently labeled, as were serosal cells and cells in the smooth muscle layers. Sham-operated intestines from animals that had been starved for at least 1 week exhibited a labeling pattern similar to that of intes-

[3] Sham operations consisted of making an incision in the abdominal wall, pulling out a loop of intestine, replacing it into the abdominal cavity without transection, and suturing closed the body wall.

tines of unoperated, well-fed and unoperated, starved animals.

No change in labeling with [^{3}H]thymidine was detected in transected intestines injected from 4 hr to 2 days after operation. The submucosa became edematous within the first 6 hr after transection and, by 10 hr, the outrolled mucosal epithelium had changed from columnar to cuboidal. By 2 days, the only frequently labeled cells were still those of the mucosal epithelium.

At 4 days posttransection, the epithelial outgrowths were in the process of moving away from the point of attachment of the everted mucosal epithelium and serosa and the blastema was beginning to form in the area around these epithelial extensions. The circular and longitudinal layers of smooth muscle separated and the circular layer became edematous in the area where eversion had taken place. Smooth muscle cells in this area underwent morphological alteration and appeared to be passing into the blastema. Smooth muscle nuclei changed from their normal, thin, elongate, spindle shape to a slightly thicker, more oval shape and exhibited a slight decrease in nuclear staining. The serosal cells that had folded back on themselves were now cuboidal with globular, rounded nuclei. These cells were labeled but those further proximal to the plane of transection were not.

By 6 days, serosal cells all along the intestine for a considerable distance proximal to the plane of transection were incorporating [^{3}H]thymidine (Figs. 1A and B and 2). The frequency of labeling in serosal cells increased from 4 to 6 days posttransection and then leveled off but remained high throughout the regeneration period. The blastema began to increase in size from 4 to 6 days and, by 6 days posttransection, some blastemal cells were labeled. Outgrowths of mucosal epithelium were present but no larger than at 4 days. Smooth muscle cells continued to undergo morphological changes and enter the blastema; mucosal epithelial cells, serosal cells, and blastemal cells were all labeling by this time. Fusion of the two free ends of the intestine had usually occurred by 6 days after transection but such connections were very superficial. The free ends were more tightly joined by 8 days.There was an increase in both the total cell number and in the number of labeled cells in the blastema during this period. Epithelial outgrowths were still present and groups of labeled epithelial cells had the appearance of advancing through the blastema.

The earliest labeling of smooth muscle cells was observed at 10 days after transection. These cells were incorporating [^{3}H]thymidine quite frequently by 12 days and numerous, labeled smooth muscle cells appeared to be moving into the blastema (Figs. 3A and B). The migrating mucosal epithelia from each end had fused by this time but blastemal cells still formed at least part of the wall of the reconstituted intestinal lumen in most cases. Serosal cells continued to be frequently labeled.

By 15 days posttransection, smooth muscle cells were still incorporating [^{3}H]thymidine. Little functional regeneration, as determined by gross observation of green bile in the lumen in the area of transection, had yet occurred. The blastema was still large and numerous blastemal cells were labeled. Five days later, 20 days posttransection, serosal cells were still labeling. In some cases, the reconstituted lumen was now completely lined with epithelium but other intestinal tissues had not yet regenerated, except for the serosa, which was usually partially complete across the area of transection. The frequency of labeling of smooth muscle had decreased by this time and the only labeled smooth muscle cells were those immediately adjacent to the blastema.

By 25 days, the epithelially lined lumen was nearly always continuous across the zone of transection as was also the case after 30 days (Fig. 4A). Blastemal cells, which were still labeling but fewer in number, were becoming aligned in preparation

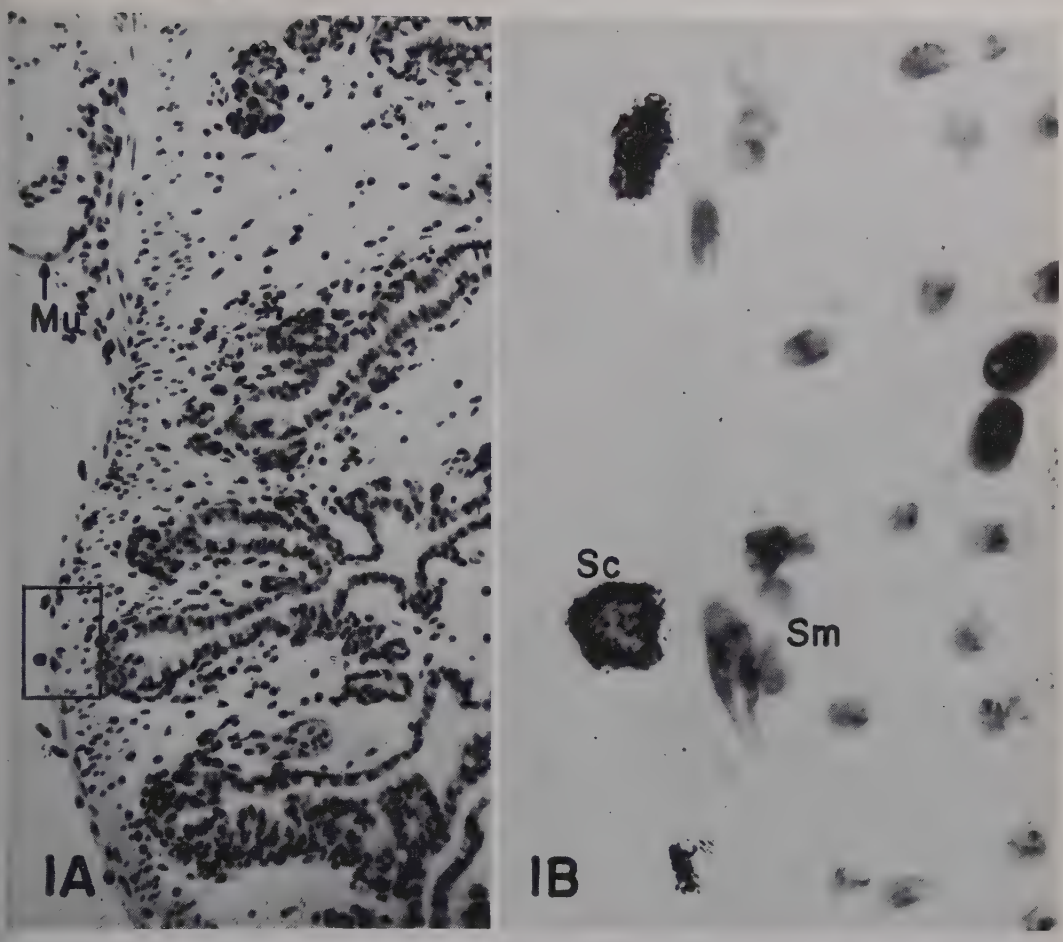

FIG. 1. (A), Autoradiograph of serosal cells near the area of attachment of everted tissues to the serosa. The animal was injected with [^{3}H]thymidine 6 days posttransection and killed 6 hr later. The main part of the intestinal lumen is to the right of the figure and at the left are seven heavily labeled serosal cells, two of which are shown at higher magnification in (B). In the upper left hand corner is the everted mucosal epithelium (Mu) in which the cells have become cuboidal. × 104. (B), Autoradiograph of serosal cells incorporating [^{3}H]thymidine. This figure is an enlargement of the area outlined in (A) and shows the intensity of labeling of the serosal cells (Sc). Smooth muscle cells (Sm), cut in both longitudinal and cross section, are present to the right of the serosal cells. × 880.

for reconstitution of the submucosa and smooth muscle layers (Fig. 4B). Giant cells, consisting of a large mass of cytoplasm containing numerous nonlabeled nuclei, first appeared in the blastema at this time. O'Steen (1958) observed such cells during regeneration of the intestine and concluded that they were characteristic of injured tissues in a state of repair.

Regeneration continued to progress and, by 30 days posttransection, labeled blastemal cells were aligned between the regenerated serosa and the mucosal epithelium. Smooth muscle cells no longer incorporated [^{3}H]thymidine except in the area where new smooth muscle was still differentiating. Unlabeled giant cells were present in increased numbers.

By 35 days after the intestine had been cut, the size of the blastema had decreased considerably. Although the lumen was completely lined by epithelium, other tissue regeneration was still in progress. Labeling of serosal cells had decreased in

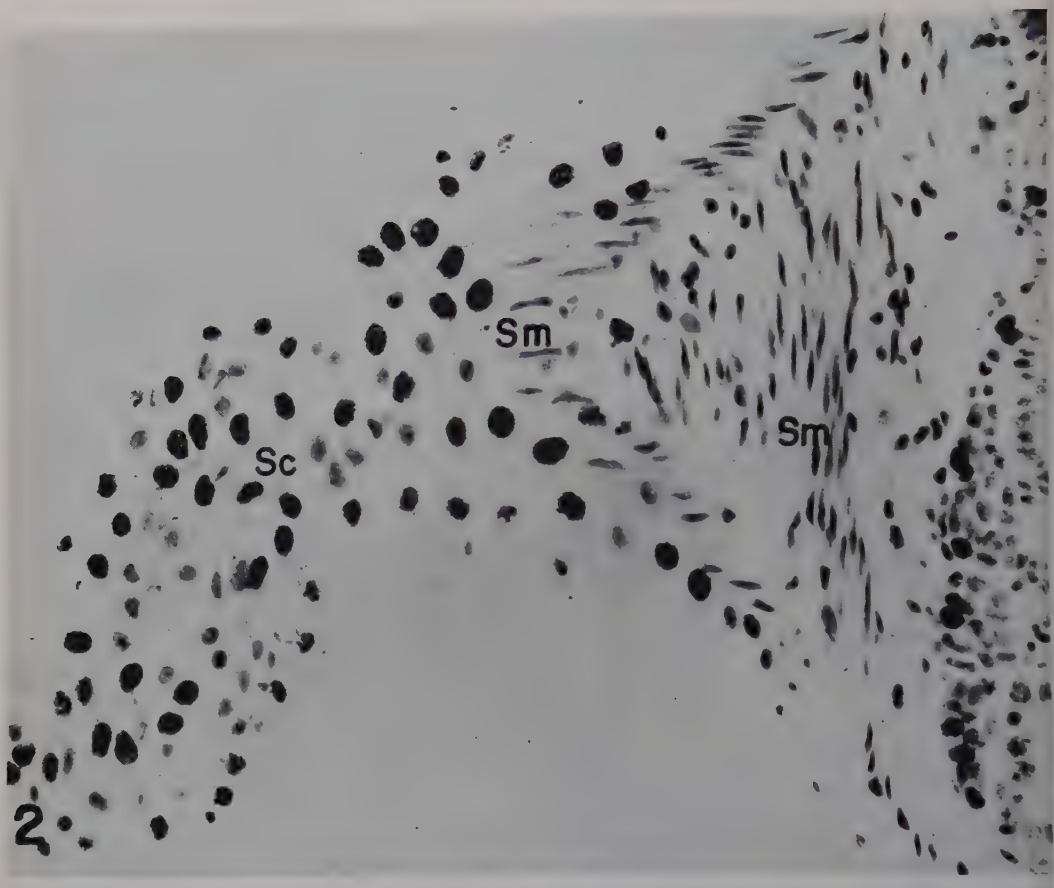

FIG. 2. Autoradiograph of surface view of serosal cells. The animal was injected with [^{3}H]thymidine 8 days after the intestine was cut and killed 6 hr later. Numerous heavily labeled, round serosal cell nuclei (Sc) are present. In the center are nonlabeled, elongate, smooth muscle nuclei (Sm) of the outer longitudinal layer and to their right are smooth muscle nuclei of the inner circular layer. Labeled mucosal epithelial cells are present at the far right. × 179.

frequency and these cells were often unlabeled in the area of the original transection. A summary of the events during intestinal regeneration is presented in Table 1 and the [^{3}H]thymidine labeling pattern of intestinal tissues during regeneration is summarized in Table 2.

DISCUSSION

This study demonstrated that tissues of the intestinal stump, outside of the mucosal epithelium, namely, smooth muscle and serosa, were activated to synthesize DNA and began incorporating [^{3}H]thymidine at specific times after transection of the intestine. In the nonregenerating intestine, most of these cells are not dividing. Since DNA synthesis is an important characteristic of internal stump cells transforming into limb blastemal cells (Hay and Fischman, 1961), this evidence suggests that several tissues of the intestine are involved in formation of the intestinal blastema. In addition, both cells of the serosal and smooth muscle layers were observed to undergo morphological alteration, therefore fulfilling the second characteristic of cells developing into blastemal cells according to Hay and Fischman (1961). From the present study it could not be determined if smooth muscle cells underwent dedifferentiation (i.e., lost their typical ultrastructural features) as has been demonstrated in smooth muscle cells

in culture (Chamley *et al.*, 1974). It should also be pointed out that perhaps some of the labeled cells in the smooth muscle layers were not smooth muscle cells at all but were connective tissue cells. Many of the labeled cells, however, on the basis of light microscopy, appeared to be the former.

In the present investigation, serosal cells and smooth muscle cells had the appearance in sections of passing into the blastema. However, labeled cells were not traced into the blastema by fixation at different time intervals after isotope injection as has been done with the internal tissues of the limb stump (Hay and Fischman, 1961). Therefore these experiments should be performed before it can be unequivocally stated that serosal cells and smooth muscle cells are passing into the blastema that forms during regeneration of the intestine. It might be possible to selectively label serosal cells and follow them by injecting [^{3}H]thymidine at 4 days

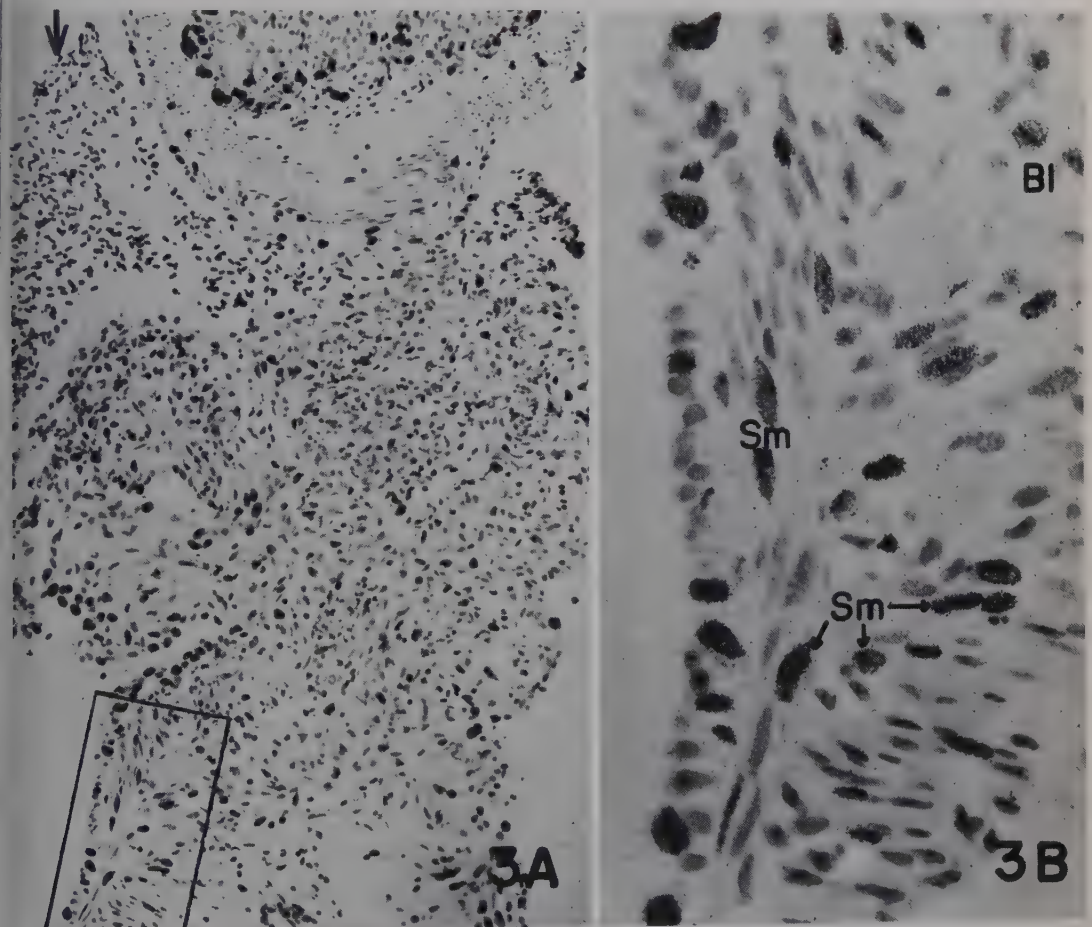

FIG. 3. (A), Autoradiograph of regenerating intestinal blastema 12 days posttransection. The animal was killed 6 hr after the injection of [^{3}H]thymidine. The blastemal mass (center) separates the two ends (top and bottom) of the intestine. A large group of red blood cells (arrow) is present at the upper left. Numerous heavily labeled smooth muscle and blastemal cells are shown some of which may be seen better at higher magnification in B. × 70. (B), Autoradiograph of labeled smooth muscle and blastemal cells from a 12-day-old regenerating intestine. This figure is an enlargement of the area outline in (A). Numerous, elongate, smooth muscle cell nuclei (Sm) cut in longitudinal and oblique planes are shown overlain by silver grains. In the upper right corner are a few, labeled, round blastemal cells (Bl). At the far left is an epithelial extension containing several labeled cells. × 300.

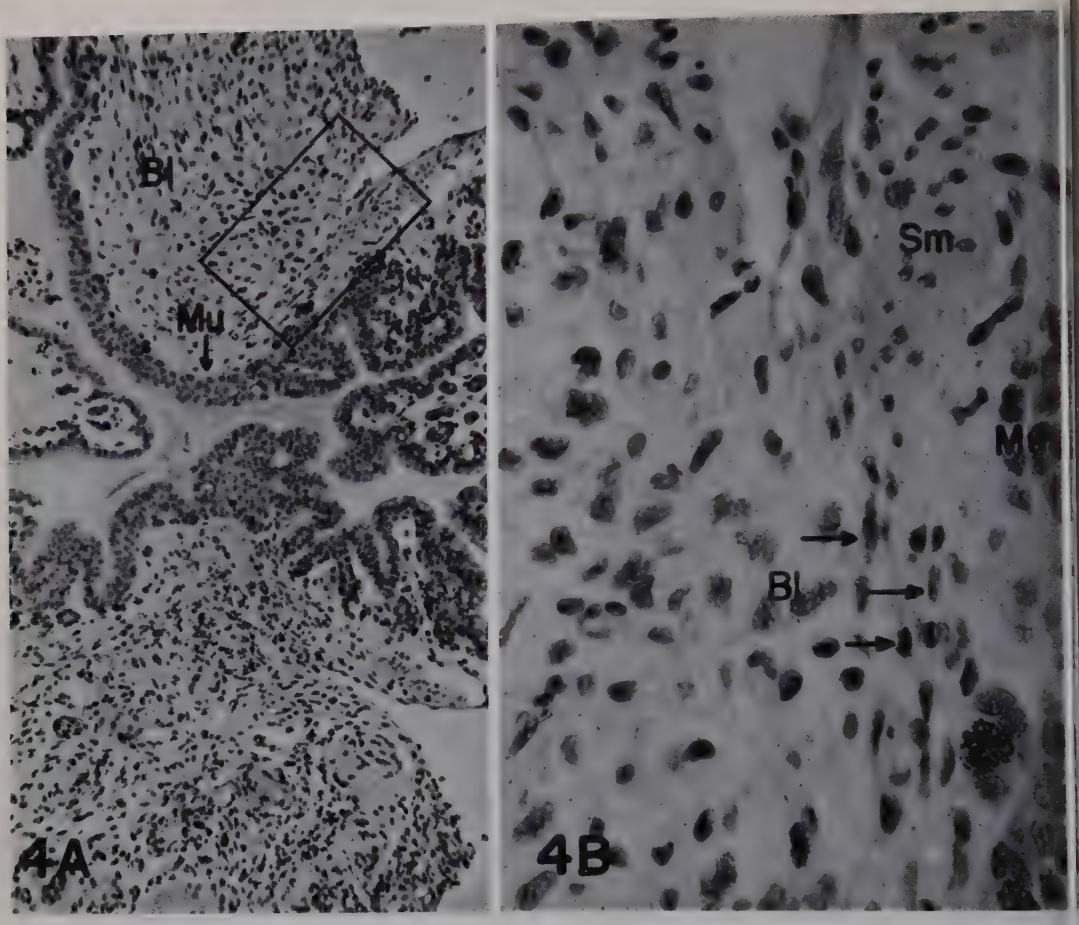

FIG. 4. (A), Autoradiograph of regenerating intestine with completely reconstituted lumen 30 days after transection of the intestine. The animal was injected with [^{3}H]thymidine and killed 6 hr later. Portions of the blastema are shown at the top and bottom of the figure. The mucosal epithelium (Mu) is stratified in the reconstituted portion and surrounded by cells of the blastema (Bl). Partial realignment of blastemal cells appears to be taking place especially in the outlined area. This is presumably in preparation for regeneration of the submucosa, smooth muscle, and serosa. The rectangular area is enlarged in (B). × 70. (B), Autoradiograph of the area of realignment of intestinal blastemal cells in preparation for regeneration of submucosa, smooth muscle, and serosa in a 30-day-old regenerating intestine. To the far right is the mucosal epithelium (Mu) and adjacent to it in the lower portion of the figure (arrows) are cells that are becoming aligned. These appear to be orienting themselves with respect to the intact smooth muscle cells (Sm) that are present in the upper right portion of the figure. Both labeled and unlabeled blastemal cells (Bl) are present. × 310.

after transection, when these cells begin incorporating isotope, and then killing the animals at various intervals thereafter. A similar experiment to label and follow smooth muscle cells by injecting the isotope at 12 days posttransection, when smooth muscle cells begin incorporating [^{3}H]thymidine, presumably would result in the labeling of serosal and blastemal cells as well as smooth muscle cells thus making it impossible to follow the smooth muscle cells alone.

The findings of this experiment, though very similar, are slightly different from those of Hay and Fischman (1961) in that the several tissues of the intestine did not all begin synthesizing DNA at the same time after transection as was the case for

the internal tissues of the limb stump. Serosal cells began labeling first at approximately 4–6 days posttransection and smooth muscle cells began later, at 12 days after transection. This suggests that the mechanisms controlling the initiation of DNA synthesis in serosal cells undergo more rapid activation upon transection of the intestine than those in smooth muscle cells. Morphological changes appeared to occur concurrently with DNA synthesis in serosal cells but preceded DNA synthesis in smooth muscle cells. In other systems, morphological dedifferentiation occurs simultaneously with DNA synthesis; these include connective tissue and skeletal muscle cells of the amputated newt limb (Hay and Fischman, 1961) and dorsal iris cells of lentectomized newt eyes (Reyer, 1971; Yamada and Roesel, 1969; Eisenberg and Yamada, 1966).

O'Steen and Walker (1962) studied the contribution of the mucosal epithelium to the blastema by injecting [^{3}H]thymidine into the animals 10 days prior to transection of the intestine in order to label these epithelial cells exclusively. The fate of these labeled cells was followed by killing the animals at various intervals after the intestine had been cut. Because silver grains were observed only over the reconstituted mucosal epithelium in regenerated intestines and since very few labeled blastemal cells were observed in these animals, and these only 20 days or more after transection of the intestine, it was con-

TABLE 1

CHARACTERISTICS OF INTESTINAL REGENERATION AT VARIOUS INTERVALS AFTER TRANSECTION OF THE INTESTINE

Days after transection of intestine	0	4[a]	12[a]	1	2	4	6	8	10	12	15	20	25	30	35
Number of operations	2	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Characteristic	Number of cases showing each characteristic														
Eversion of tissues at transected ends	2	3	2	3	3	3	3	3	3	3	3	3	3	3	3
Attachment of everted tissues to serosa	0	0	0	0	2	3	3	3	3	3	3	3	3	3	3
Formation of epithelial extensions	0	0	0	0	1	3	3	2	3	3	3	3	3	3	3
Presence of blastemal cells	0	0	0	0	0	3	2	3	3	3	3	3	3	3	3
Fusion of transected ends	0	0	0	0	1	2	2	3	3	3	3	3	3	3	3
Morphological alteration of serosal cells	0	0	0	0	0	3	3	3	3	3	3	3	3	2	3
Morphological alteration of smooth muscle cells	0	0	0	0	0	3	2	1	2	3	3	1	2	0	0
Partial fusion of epithelial extensions	0	0	0	0	0	0	1[b]	1	3	2	2	0	0	1	0
Formation of complete lumen lined by epithelium	0	0	0	0	0	0	0	0	0	1	0	2	2	2	3
Evidence for regeneration of other tissues of intestine	0	0	0	0	0	0	0	0	1[c]	0	0	1[c]	0	1	1
Presence of giant cells	0	0	0	0	0	0	0	0	0	0	1	2	1	2	2

[a] Hours.

[b] Epithelial extension fused to lateral border of opposite end.

[c] Serosa regenerated on one side only.

TABLE 2

[^{3}H]THYMIDINE LABELING OF CELLS DURING REGENERATION OF THE INTESTINE

Days after transection of intestine	Number of operations	Number of intestines containing labeled cells in various tissues			
		Mucosal epithellium	Serosa	Smooth muscle	Blastema
0	2	2	0	0	0
4[a]	3	3	0	0	0
12[a]	3	3	0	0	0
1	3	3	0	0	0
2	3	3	0	0	0
4	3	3	2	0	0
6	3	3	2	0	1
8	3	3	3	1	3
10	3	3	3	0	3
12	3	3	3	3	3
15	3	3	3	3	3
20	3	3	3	3	2
25	3	3	3	2	3
30	3	3	1	1	3
35	3	3	1	2	2

[a] Hours.

cluded that mucosal epithelial cells did not contribute significantly to the blastema. O'Steen (1958) had previously claimed that some mucosal epithelial cells changed into blastemal cells. The present study contributed no additional evidence for this question since epithelial cells of the intestine are dividing and labeling at all times both before and after transection of the intestine. Therefore, the onset of incorporation of [^{3}H]thymidine in epithelial cells could not be used as a criterion for dedifferentiation. It seemed that the principal function of the mucosal epithelium was that of migration through the blastemal mass towards the epithelium of the opposite end. Morphological alterations in the columnar, absorptive cells of the surface epithelium were observed at the transected ends of the intestine. They changed from columnar to cuboidal cells and remained this way throughout the period of migration. No significant increase in labeling of the cuboidal epithelial cells was noted at any time during regeneration and only after the lumen had regenerated did they return to their normal, columnar shape.

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Regulation of Acid Phosphatase Activity in the Ovary of *Drosophila melanogaster*

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Acid phosphatase (Acph) activities and protein content were measured in developing ovaries of adult flies. Acph and protein increased approximately logarithmically for the first 2 days of adult life and then plateaued at about 80 and 35 times, respectively, the levels present at eclosion. The specific activity of Acph was constant for the first 15 hr and then increased by a factor of three over the next 2 days. Analysis of staged follicles showed that the specific activity of Acph starts to increase at stage 10. Ovaries from homozygotes for *Acph-1*^{n4}, a null activity mutant, showed constant low specific activity, indicating that this gene codes for the major ovarian Acph. Ovarian transplantations between *Acph-1*^{n4} and wild type showed that Acph is made by the ovary. Ovaries from isolated abdomens failed to increase in Acph activity or protein, but treating isolated abdomens with ZR-515, a juvenile hormone analog, caused nearly normal levels to be attained. Ovaries of the female sterile mutant *ap*^{4} failed to develop Acph activity unless they were implanted into a normal host or treated with ZR-515. Ovaries from the female sterile mutant *fs(3)L3* developed no increase in Acph activity even when treated with ZR-515. The results demonstrate that the activity of a genetically localized enzyme is controlled by a chemically defined hormone in a genetically favorable higher eucaryote.

INTRODUCTION

During the development of a eucaryote, different tissues accumulate a distinct set of proteins due to differential gene activity. Gene activity can be controlled by small effector molecules such as hormones (Tomkins *et al.*, 1969) or by the activity of other genes (Lewis, 1964). Investigation of both gene–gene and gene–effector interactions in the same system is facilitated if the system has the following attributes. First, the organism should be readily manipulated genetically so that the genes specifying structural proteins as well as the genes responsible for regulatory functions can be identified, mutated, and genetically and cytogenetically localized. Second, the tissue should possess reasonable amounts of material so it is suitable for biochemical analysis. Third, the appearance of specific proteins should be regulated by chemically defined effector molecules. The present work represents an effort to develop a system that satisfies these three criteria.

The first criterion is met by *Drosophila melanogaster*, the only higher eucaryote whose genetics can be easily manipulated (Lindsley *et al.*, 1968). The second requirement is satisfied by the choice of fly ovaries as a tissue to study. The ability of flies to lay 60% of their weight per day in eggs (King *et al.*, 1955; David *et al.*, 1968) indicates the prodigious biosynthetic activity involved in egg production. Finally, the inducer of ovarian maturation, the juvenile hormone, has been identified and chemically characterized (Doane, 1973). Therefore it seemed appropriate to look for specific ovarian enzymes whose activity was dependent on juvenile hormone. We report here that acid phosphatase (Acph) is such an enzyme.

In insect ovaries, Acph appears to play a role in yolk metabolism (Lockshin, 1969), and fly ovaries and eggs contain high levels of Acph (Yao, 1950; Giorgi, 1974). The structural gene for this enzyme has been identified by MacIntyre (1966), and null

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activity mutants are available (Bell *et al.*, 1973). Furthermore, it has been shown that a juvenile hormone analog (JHA) can induce yolk formation in an otherwise nonvitellogenic female sterile mutant in *Drosophila* (Postlethwait and Weiser, 1973). In this report we exploit these facts to find whether Acph activity is controlled in ovaries by JHA and to look at two mutants affecting Acph regulation.

MATERIALS AND METHODS

A. Animals and hormones. Normal ovaries were obtained from wild-type flies homozygous for electrophoretic variant *Acph-1*^{C} (3-101.4), and heterozygous *ap*^{4}*/SM5* females. Flies with low Acph activity were homozygous for the null activity allele *Acph-1*^{n4}. Two nonvitellogenic female sterile mutants were examined. The newly discovered female sterile mutation *fs(3)L3* when homozygous fails to produce yolky oocytes (Postlethwait and Landers, unpublished). Homozygous *apterous-four* (*ap*^{4}, 2-55·2) animals, in addition to being nonvitellogenic, are also wingless and short lived (Butterworth *et al.*, 1965). The balanced stocks *fs(3)L3/TM2* and *ap*^{4}*/SM5* provided homozygous female steriles. See Lindsley and Grell (1968) for further explanation of genotypes. Animals were cultured using standard techniques on cornmeal–agar–molasses–yeast medium.

To provide flies of known ages, females eclosing over either a 1- or 2-hr interval were incubated at 25°C without males for specified periods of time. To test for tissue autonomy, ovarian transplantations were performed as described by Ursprung (1967), using as hosts and donors adult flies less than 2 hr old. Larval fat body cells were removed from all ovaries before transplantation. Ovaries were dissected from hosts 48 hr after eclosion and immediately assayed for Acph. Data from 149 transplants are reported in this paper.

In order to have animals lacking the corpus allatum, the source of juvenile hormone (Doane, 1973), animals were ligated between the thorax and abdomen with the hair of a blond child, and the fly's head and thorax were removed. Isolated abdomens were maintained on filter paper soaked with *Drosophila* Ringer's (Ephrussi and Beadle, 1936) to which a few crystals of penicillin, streptomycin, and phenylthiourea were added. Forty-eight hours later, preparations that demonstrated muscular activity were dissected and their ovaries analyzed.

Animals were treated with approximately 0.15 μg of the juvenile hormone analog ZR-515 (isopropyl-11-methoxy-3,7, 11-trimethyldodeca-2,4-dienoate) dissolved in 0.3 μl of Mallinckrodt Nanograde acetone. Hormone was topically applied to the ventral abdominal integument 2 hr after ligation. About 2500 ovaries were individually dissected from flies and assayed in the experiments reported here.

B. Acid phosphatase and other assays. Two Acph assay procedures were employed, one for transplants (without protein assay) and the other for developmental profiles (with protein assay). The Acph activity in transplants was assayed by homogenizing the sample ovary in 0.050 ml of 0.01 *M* sodium citrate, 0.01 *M* Tris–HCl (Sigma Trizma), 0.15 *M* KCl (pH 4.7) in a glass homogenizer (Kontes Glass Co.). At time zero, 0.050 ml of substrate (5 m*M* *p*-nitrophenylphosphate, Sigma, in homogenization buffer) was added, and after 60 min at 23°C the reaction was terminated with the addition of 0.300 ml of 0.05 *M* sodium borate (pH 8.4). (At pH 8, the reaction proceeds at less than 1/10 the rate of maximum activity, at pH 4.4, and the product, *p*-nitrophenol, is yellow.) Immediately after termination the optical density of the product *p*-nitrophenol was then measured at 400 nm on a Cary 14 spectrophotometer.

The enzyme assays for developmental studies were modified to permit protein assay of a sample after it had been analyzed for enzyme. In this case the homogenization buffer was 0.1 *M* sodium citrate (pH 4.5) and the termination buffer was 1

M Tris–HCl with 0.1% sodium dodecyl sulfate (pH 7.5). To accommodate ovaries of widely different enzyme activities, between one and twelve ovaries per sample were used and incubation time varied between 25 and 75 min. For the incubation times and sample sizes used the enzyme assay was linear with time and with tissue amount. After the enzyme assay, protein was determined according to the following procedure, modified from Schaffner and Weissman (1973). Protein was precipitated with 0.15 ml of 60% trichloroacetic acid. The protein was absorbed to a Millipore filter, stained with amido schwarz, and eluted according to Schaffner and Weissman (1973). Absorbance at 630 nm was recorded and compared to bovine serum albumin (Sigma) standards. The protein assay was linear between 1 and 20 μg of bovine serum albumin. All chemicals were reagent grade obtained from commercial suppliers, except where otherwise noted.

RESULTS

A. Developmental Profile of Ovarian Acid Phosphatase Activity

The change in Acph activity during normal ovarian maturation is shown in Fig. 1. For the first 40 hr after eclosion, wild-type *Acph-1*^{C} ovaries and phenotypically normal ovaries of *ap*^{4}/*SM5* heterozygotes show an approximately logarithmic increase with time of both Acph activity (Fig. 1A) and protein content (Fig. 1B). Thereafter both enzyme activity and protein content remain constant until at least 80 hr. Between eclosion and 40 hr, Acph activity per ovary increases by a factor of about 80, while protein per ovary increases by a factor of 35. Therefore the increase in specific activity of Acph (Fig. 1C) is more modest than the dramatic increase in absolute activity noted in the ovary in the first 2 days. Figure 1C shows that in the genotypes with normal ovaries, *Acph-1*^{C} and *ap*^{4}/*SM5*, the specific activity is constant during the first 15 hr, and then increases about threefold during the next 2 days. This time lag argues that an increase in enzyme activity is not solely a result of an increase in general protein.

B. Acid Phosphatase Activity as a Function of Follicular Stage

To find the follicular stage at which Acph specific activity begins to increase, we dissected out groups of follicles at defined follicular stages, and assayed them for enzyme and protein. Figure 2 shows that between the previtellogenic stage 7 and the mature stage 14, Acph activity increases to the same degree as whole ovaries between eclosion and 40 hr (see King (1970) for staging characteristics). An increase in specific activity occurs at stage 10, and it takes about 15 hr to go from stage 7 to stage 10 (King, 1970). This apparently explains the 15-hr lag in specific activity observed in whole ovaries (Fig. 1C). Therefore, it can be concluded that recruitment of new follicles is not necessary to account for the Acph increases observed, but Acph increases can be accounted for solely by increases in enzyme activity in individual follicles.

C. Identification of the Structural Gene for Ovarian Acid Phosphatase

In order to find whether the increase in Acph activity observed during development was due to enzyme coded for by the structural gene identified by MacIntyre (1966), we examined ovarian enzyme in the null allele *Acph-1*^{n4}. In general, enzyme activity (Fig. 1A) was about one-tenth) that found in normal ovaries. This residual activity may be due to phosphatases coded for by genes other than *Acph-1*. Although Acph activity is low in *Acph-1*^{n4}, total ovarian protein increase follows a normal time course (Fig. 1B). The specific activity in *Acph-1*^{n4} ovaries (Fig. 1C) is unchanged over the time period examined, showing that phosphatase activity coded for by genes other than *Acph-1* increases

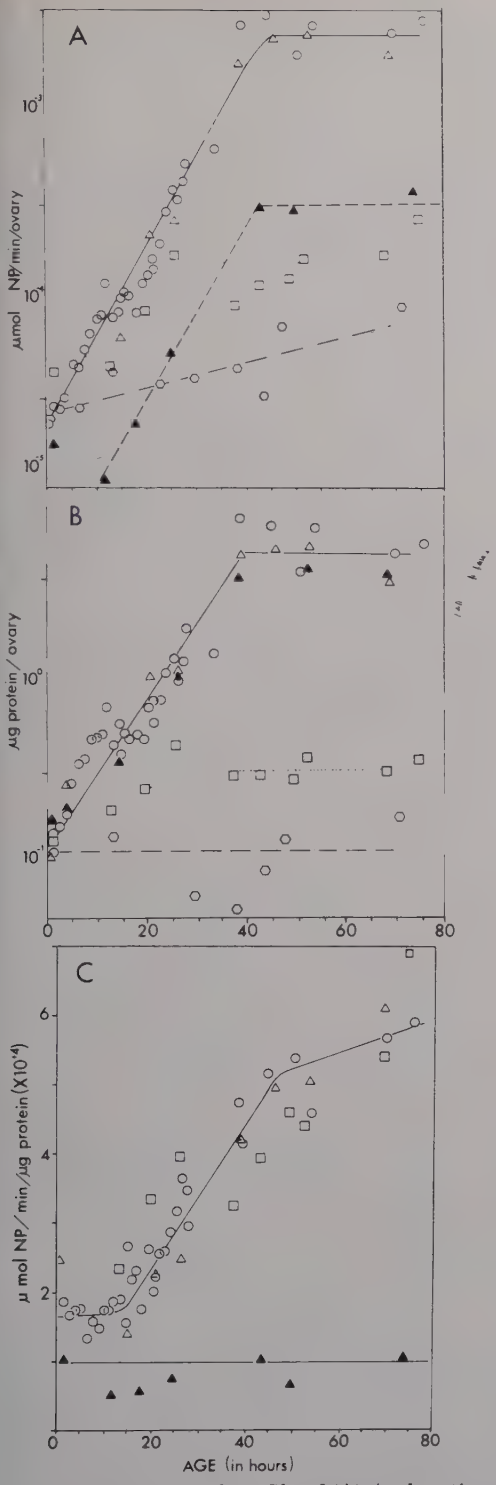

FIG. 1. Developmental profile of (A) Acph activity; (B) protein content; and (C) specific activity in the ovaries of five genotypes. Points below 2×10^{-3} μmol of *p*-nitrophenylphosphate (NP) are based on a mean of 8.3 ovaries per point; points above this level are based on two ovaries per point. For each genotype the developmental profile was examined in three separate runs. The absolute values at each age varied between runs, due perhaps to differences in larval or adult nutrition. However the shape of the curves were all the same and so data are shown for only one run. *Acph-1*^{C}, open triangles; *Acph-1*^{n4}, filled triangles; *ap*^{4}/*SM5*, open circles; *ap*^{4}, open squares; *fs(3)L3*, open hexagons.

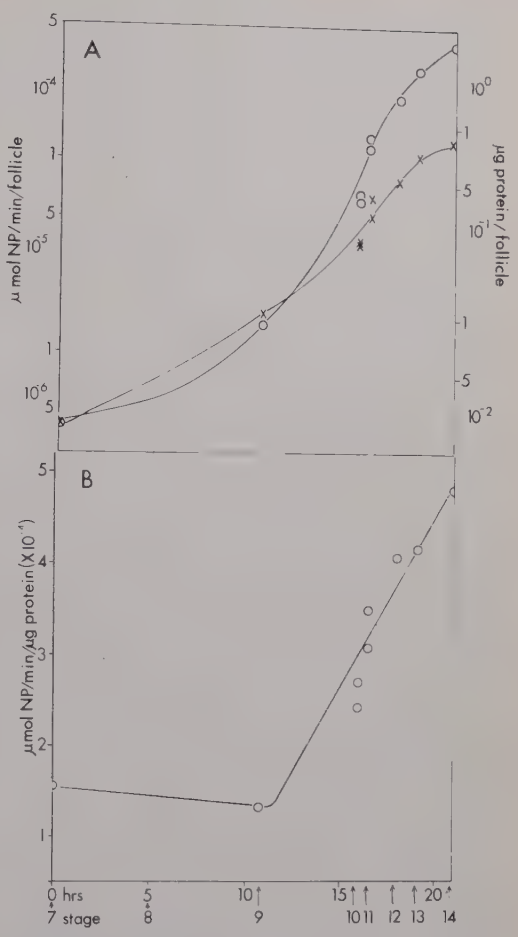

FIG. 2. (A), Acph activity and protein content, and (B), specific activity as a function of follicular stage. Each point represents a mean of 10.0 follicles dissected out individually. This experiment was repeated three times on separate occasions; the data of a typical replicate are shown. In (A), an open circle represents Acph activity and a × represents protein content. On the abscissa the upper numbers indicate hours after eclosion and the lower numbers indicate the developmental stage of the follicle.

coordinately with total protein. These data show that *Acph-1* is the major gene responsible for acid phosphatase activity in the ovaries.

D. Site of Ovarian Acid Phosphatase Synthesis

Having determined that Acph activity per ovary dramatically increases during the first 2 days of adult life, we next needed to test whether the ovary synthesizes the Acph found in eggs. This is necessary since *Drosophila* ovaries can sequester proteins from the hemolymph (Mahowald, 1972). To examine this question, ovaries were transplanted from flies genetically lacking Acph into the abdomens of flies with normal activity and vice versa. The implanted ovaries mature along with those of the host, although they do not connect to the host's genital ducts. This allows the implanted ovary to be distinguished from the host's ovaries. To overcome the fact that donor ovaries do not oviposit and to avoid contamination of donor tissue by host tissue, mature oocytes were dissected from donor or host ovaries and then assayed for enzyme. Mature stage 14 oocytes dissected from *Acph-1*^{C} wild-type females contain ten times more enzyme activity than *Acph-1*^{n4} oocytes (Fig. 3). In control experiments where *Acph-1*^{C} or *Acph-1*^{n4} ovaries were implanted into hosts of the same genotype, the Acph activity of eggs from both host and donor ovaries were the same as unoperated controls. In the experimentals, *Acph-1*^{C} ovaries implanted into *Acph-1*^{n4} hosts behaved autonomously: Oocytes from donor ovaries had high enzyme activity while oocytes from host ovaries had low activities. Results for the reciprocal experiment confirmed the autonomy of ovaries (Fig. 3). Thus, the genotype of the ovary determines its phenotype independent of the host. From this result, and the fact that *Acph-1* is the structural gene for this enzyme, it can be concluded that an ovary synthesizes the Acph found in its oocytes.

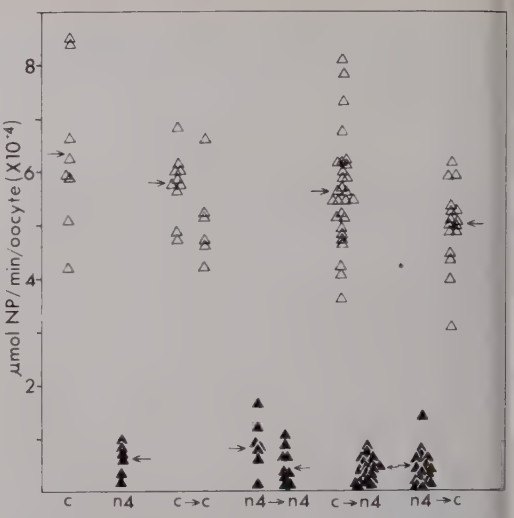

FIG. 3. Acph activity of oocytes from ovarian transplants. Each n^4 point (*Acph-1*^{n4}, filled triangles) represents a mean of 6.3 oocytes per sample, and each C point (*Acph-1*^{C}, open triangles) represents a mean of 7.3 oocytes per sample. C → n^4 means an *Acph-1*^{C} ovary implanted in an *Acph-1*^{n4} host. These data were obtained from 63 transplants. Arrows indicate means.

E. Extraovarian Factors Inducing Acph Activity

To test whether extraovarian factors control ovarian Acph levels, the abdomens, containing the ovaries, were separated from the anterior neuroendocrine centers by a ligature, and then the heads and thoraces were removed. In general these abdomens pigmented normally. Table 1 shows that ovaries in isolated abdomens failed to develop Acph activity or to increase in protein. These ovaries also had no vitellogenic follicles, and larval fat body failed to degenerate. This result indicates that some factor derived from the head or thorax is necessary for ovarian acid phosphatase levels to increase. When isolated abdomens were treated with JHA, their ovaries formed mature stage 14 oocytes and larval fat body disappeared. Furthermore, Acph activity increases by a factor of about 40, and protein content increased by a factor of about 10 (Table 1). The specific activity in these preparations was larger than that normally found at 48 hr. These experi-

ments prove that JHA can substitute for the natural inducer missing from ligated abdomens.

F. Acid Phosphatase Activity in ap⁴

To initiate a search for control mutants affecting ovarian Acph activity, enzyme and protein levels were determined for the female sterile mutant *ap⁴*. Ovaries from *ap⁴* animals show a delayed increase in Acph and protein content compared to normal ovaries, and final levels reach only 7% of normal (Fig. 1). Despite the failure of *ap⁴* ovaries to mature, the specific activity of Acph follows a normal time course.

To test whether extraovarian factors control ovarian Acph levels in *ap⁴*, we transplanted ovaries from homozygous *ap⁴* flies into their normal *ap⁴/SM5* heterozygous siblings and vice versa, along with control transplantations. The results (Fig. 4) contrast sharply with those from the *Acph-1^n4* transplants. While *ap⁴* ovaries have low Acph activity after 48 hr of culture in an *ap⁴* host, they develop considerable enzyme activity when implanted into a heterozygous host, whether it is male or female. This confirms the observation that *ap⁴* ovaries are nonautonomous (King *et al.*, 1965). Heterozygous ovaries are also nonautonomous: *ap⁴/SM5* ovaries fail to increase in Acph activity when cultured in an *ap⁴* host but develop normally in a heterozygous host. These experiments permit the conclusion that a factor that can be supplied by male and female heterozygous flies and that is lacking in *ap⁴* flies controls ovarian Acph activity and total protein.

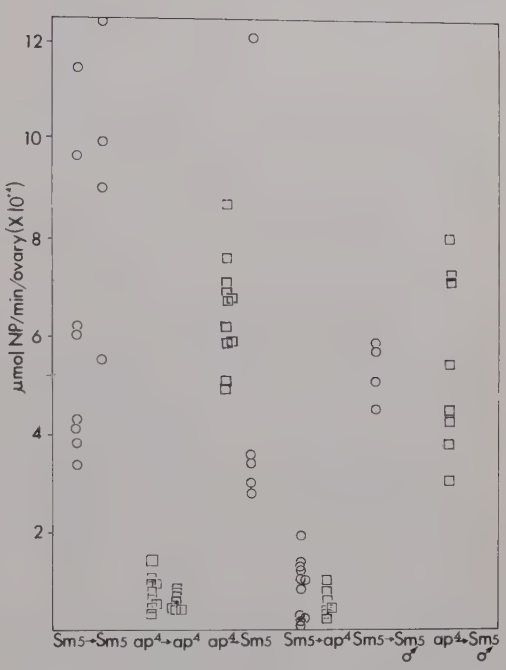

FIG. 4. Acph activity of *ap⁴* ovarian transplants. *ap⁴*, squares; *ap⁴/SM5*, circles, SM5 → ap⁴ means an *ap⁴/SM5* ovary implanted into an *ap⁴* host. Each point from an *ap⁴/SM5* host represents a mean of 1.7 ovaries per sample. Each point from an *ap⁴* host represents a mean of 4.8 ovaries per sample. These data were obtained from 86 transplants.

TABLE 1

ACID PHOSPHATASE ACTIVITY IN OVARIES FROM ISOLATED ABDOMENS

Preparation	Age (hr)	Genotype	Number of determinations	Average number of ovaries per determination	NP[a] [(μmol/min/ovary) (× 10⁴)]	Protein (μg/ovary)	NP[a] [μmol/min/μg of protein) (× 10⁴)]
Unligated control	1	*Acph-1^C* and *ap⁴/SM5*	6	9.8	1.05	0.443	2.37
Unligated control	48	*Acph-1^C* and *ap⁴/SM5*	8	2.1	77.2	15.0	5.44
Ligated control	48	*Acph-1^C*	19	5.6	0.86	0.550	1.56
Ligated + JHA	48	*Acph-1^C*	19	1.9	28.9	3.87	7.46

[a] NP, *p*-nitrophenylphosphate.

To test whether juvenile hormone might be the factor supplied to ap^4 ovaries by a normal host we treated ap^4 and $ap^4/SM5$ flies with JHA at eclosion. At intervals thereafter their ovaries were dissected and assayed for protein and Acph. JHA does not seem to alter the normal development of enzyme activity (Fig. 5A) and protein content (Fig. 5B) in phenotypically normal $ap^4/SM5$ ovaries. In contrast, JHA causes a significant although variable increase in protein content per ovary of mutant ap^4 ovaries. Both enzyme and protein approach normal levels in hormone-treated mutant ovaries. The specific activity in juvenile hormone analog-stimulated ap^4 is greater than normal (Fig. 5C). This result reinforces the idea that the increase in acid phosphatase activity is not merely due to an increase in general protein content. These experiments indicate that ap^4 ovaries contain low levels of acid phosphatase due to aberrant production or metabolism of the normal control agent by the fly.

G. Acid phosphatase in fs(3)L3

Ovaries from *fs(3)L3* females never develop vitellogenic eggs and they have very low enzyme and protein levels which change little in the first 3 days of life (Fig. 1). The mutant *fs(3)L3* responds differently than ap^4 to JHA. Neither protein nor enzyme levels are caused to increase in *fs(3)L3* ovaries after treatment with JHA (Fig. 5). Thus, while ap^4 and *fs(3)L3* have gross similarities in their ovarian phenotypes, they affect different steps in the control of vitellogenesis and Acph activity.

DISCUSSION

This communication reports experiments which show that the activity of acid phosphatase produced by the structural gene *Acph-1* at locus 101.4 on the third chromosome in *Drosophila melanogaster* can be controlled by a juvenile hormone analog. This is the first reported case of the induction of enzymatic activity due to a localized gene by a hormone in *Drosophila*. The Acph activity per ovary increases by a factor of 80 during the first 2 days of adult life. For the initial 15 hr after eclosion the ratio of acid phosphatase activity to total ovarian protein remains constant, but over the next 2 days the specific activity increases by a factor of about three. Flies with an inactive Acph due to a structural gene mutation ($Acph\text{-}1^{n4}$) show normal increases in total ovarian protein but constant low specific activities. Therefore the gene *Acph-1* is the structural gene for the major ovarian Acph. Transplantation of ovaries between the null allele and wild type proved that Acph in oocytes is made by the ovary. Histochemical evidence indicates it is made by the nurse cells and oocyte (Giorgi, 1974). This is in contrast to the bulk of egg protein. Fifty to eighty percent of total egg protein is present in one or two electrophoretic bands (Gelti-Douka *et al.*, 1974). Therefore, the increase in specific activity would be more dramatic if expressed as a function of ovarian synthesized protein rather than total ovarian protein.

Since corpora allata, the source of juvenile hormone, can induce vitellogenesis in *Drosophila* (Vogt, 1941, 1947; Bodenstein, 1947), it seemed reasonable that juvenile hormone might be the factor which caused Acph activity to increase in fly ovaries. The ovaries in isolated abdomens, which lack the corpus allatum and other anterior neuroendocrine organs, fail to increase in enzyme activity. Yet nearly normal Acph activities could be regained by treating isolated abdomens with a JHA. This experiment allows the conclusion that juvenile hormone causes Acph activity to increase in *Drosophila* ovaries. The specific activity of ovaries from JHA-induced isolated abdomens is greater than that which occurs normally. Apparently isolated abdomens, which do not feed, deplete protein reserves rapidly so that the normally huge amounts of yolk protein are not produced by the fat body, while the small amounts of Acph protein produced by the ovary are affected

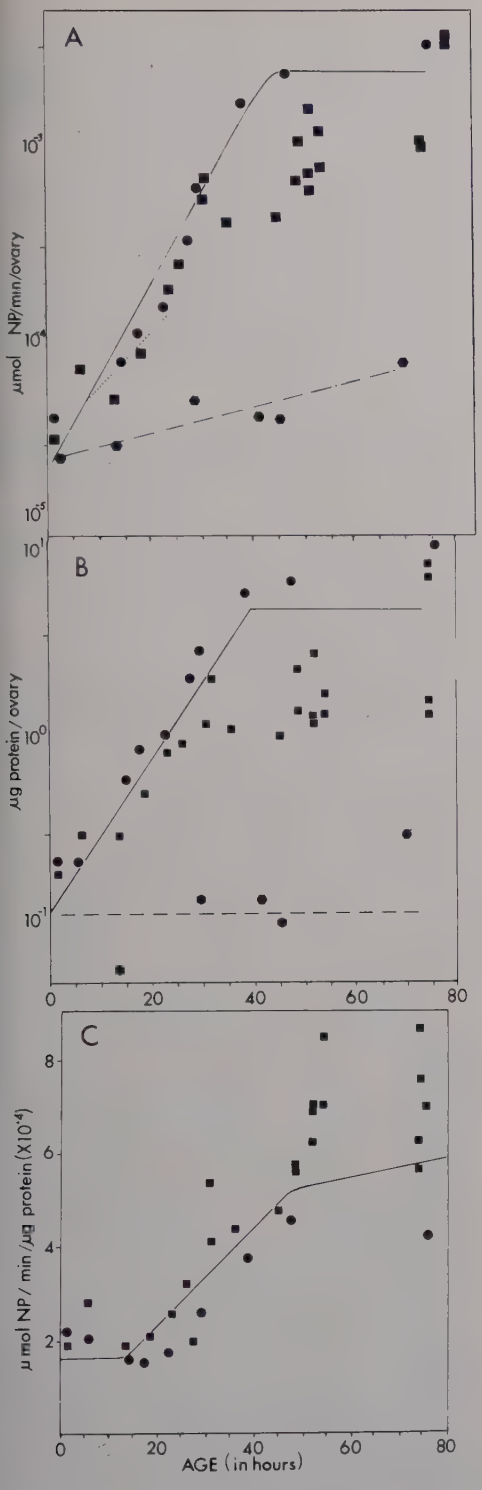

FIG. 5. (A) Acph activity; (B) protein content; and (C) specific activity of ovaries from normal and JHA-treated animals of three genotypes. $ap^4/SM5$ treated, filled circles; ap^4 treated, filled squares; *fs(3)L3* treated, filled hexagons. The non-JHA-treated control values from Fig. 1 are indicated by a solid line for wild type, a dotted line for ap^4, and alternating dashes and dots for *fs(3)L3*. For each genotype and for both hormone-treated and untreated animals, developmental profiles were examined three times. Data from a typical replicate are plotted. Each point represents an average of 5.9, 4.8, and 8.7 ovaries for ap^4, $ap^4/SM5$, and *fs(3)L3*, respectively.

to a lesser extent. Experiments with ligated animals rule out the possibility that the action of JHA is mediated by the corpus allatum as occurs in some cases (Riddiford *et al.*, 1972). But it still is not clear whether JHA acts directly on the ovary. It is yet to be determined whether juvenile hormone acts directly on the control system for acid phosphatase activity or if the hormone sets off a specific program of development that incidentally includes an increase in phosphatase activity. Whether the increase in enzyme activity is due to protein synthesis can be resolved by experiments with inhibitors of nucleic acid synthesis and protein synthesis and with antibodies made to *Drosophila* Acph.

As a prelude to a search for control mutants affecting Acph inducibility, two female sterile mutants were analyzed. Normally these mutants neither form yolky oocytes nor develop normal Acph activity (Fig. 1). However, if one of these mutants (ap^4) is treated exogenously with JHA, its ovaries develop nearly normal enzyme activity (Fig. 5). Therefore this mutant has low enzyme levels due to unavailability of inducer. Ovaries from the second female sterile, *fs(3)L3*, do not respond to JHA by an increase in enzyme or protein (Fig. 5). The extraovarian requirements for normal development are present in *fs(3)L3* since ovaries from wild-type *Oregon R* flies or ap^4 flies will develop normally when implanted into an *fs(3)L3* host (Postlethwait, unpublished). It is clear from these experiments that *fs(3)L3* and ap^4 affect different steps in the induction of Acph. Further analysis of female sterile mutants may dis-

close some in which Acph increases to normal levels but total ovarian protein levels fail to increase. A screen has been initiated for control mutants closely linked to Acph-1 which eliminate enzyme from eggs but which do not affect enzyme titers in other tissues in the hopes of testing models for gene control in eucaryotes (Britten *et al.*, 1969; Williams *et al.*, 1971).

Note added in proof: Experiments measuring cross reacting material to antibody prepared against partially purified Acph show that the increase in ovarian Acph activity is due to *de novo* synthesis of the enzyme (Sawicki, J. and MacIntyre, R. *The Isozyme Bulletin* 8, 35c–35d (1975) and Anastasia-Sawicki, J. *Genetics* 79, s9 (1975)).

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A Model for Genetic Analysis of Programmed Gene Expression as Reflected in the Development of Membrane Antigens

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The red blood cell antigen determined in mice by the *H-2*^{b} allele was detectable by hemagglutination at birth, *H-2*^{b}*-early* (bE), in strain C57BL/10, whereas that determined by *H-2*^{a} not until 3 days later, *H-2*^{a}*-late* (aL), in strain A. Each F_1 individual was both aL and bE. Timing genes segregated in the F_2 and BC_1 generations to give *H-2*^{a}*-early* (aE) and *H-2*^{b}*-late* (bL) in combinations with aL and bE in apparent Mendelian ratios. In some strain combinations in which *H-2* alleles and strain background had been interchanged, timing type was transferred along with H-2, whereas in others it was the background which seemed to be more important for the timing type. Moreover, no recombinant types were found in the F_2 and BC_1 generations of these lines. Lines originating from the (B10 × A)F_2 generation were selected for reversed parental-strain-type phenotypes under a full-sib mating regime. True breeding aE or bL lines were not realized until the ninth to eleventh inbreeding generation. We propose a *remote-cis-effect* model consisting of one temporal gene tightly linked with *H-2* and at least two others with special *cis* and recognition properties linked with each other but not with *H-2*.

INTRODUCTION

When the problem of regulation of gene action is considered as the basis of cell differentiation and morphogenesis, the room for regulatory events cannot be confined to that between the activation and translation of a structural gene. The concept has to account not only for gene activity versus nonactivity but also for the selectivity, rate, time and site of activity. Paigen (1971) postulated three classes of genes controlling such aspects of activity of structural genes for enzymes and described a number of mutations supposedly concerning "regulatory," "architectual" and "temporal" genes. Similar examples should be looked for also in other model systems. For one, histocompatibility (*H*)-gene systems might be suitable candidates for a number of reasons: The products are detectable relatively easily as antigens on intact cells of many different tissues including tumors; the antigen–antibody reaction is fairly specific and quantitative; a refined genetic and advanced chemical analysis is available for the most studied representatives of these systems (such as the mouse *H-2* system), and the numerous genetic variants can be advantageously studied on a common genetic background by means of existing congenic strains. There admittedly may be also some disadvantages of H-antigens as markers of gene activity, the main disadvantage being that because of their unknown physiological function they may have to be detected in a somewhat indirect way, on the basis of their capacity to react with specific antibodies. This, however, seems to be technical rather than any fundamental problem.

In the present study an attempt was made to analyze a known interstrain difference in the developmental time of appearance of H-2 antigens on neonatal erythrocytes (Möller and Möller, 1962) which might be due to variant forms of one or more "temporal" genes.

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Erythrocytes are agglutinable at the time of birth in strain C57BL/10 (B10), while not until 3 days later in strain A. From a number of ancillary experiments (Boubelík and Lengerová, 1975) the conclusion was reached that the early versus late appearance of agglutinability of erythrocytes by H-2 antibodies reflects the timing of appearance in a proper form of H-2 antigens rather than some other possible developmental aspects.

Briefly, there was no correlation between the average size of erythrocytes at birth and their agglutinability; if larger size reflects a less advanced stage of maturation from the generally larger blasts, the lack of correlation indicates that late agglutinability cannot be accounted for by immaturity. Furthermore, erythrocytes such as those of A-strain origin (which display late neonatal agglutinability) are better agglutinable in adult life than the early agglutinable B10 strain erythrocytes (Stimpfling and Snell, 1962; Herberman and Stetson, 1965). Thus, early agglutinability at birth may be associated with poor adult agglutinability and vice versa. Cells of some of the late agglutinable strains have also a higher density of H-2 sites on their surface (Hilgert and Kristofová, 1971). As shown also later in this paper, the agglutinability at birth is independent of the number of antigenic specificities the employed antiserum can detect: With the anti-b reagent, which detects fewer specificities, the agglutinability may be early whereas with the anti-a, which detects multiple specificities, the agglutinability may be late.

A possible involvement of an extracellular control mechanism was also ruled out by demonstrating that in allogenic radiation chimeras created reciprocally from late and early strain mice (used as irradiated recipients and donors of curative cell inocula) the appearance of donor-specific H-2 agglutinability conforms to the donor's timing type being unaffected by the contrasting host's environment. The delayed agglutinability in a late strain was further shown to concern not only neonatal erythrocytes but also those recruited from stem cells in "adult" bone marrow. Thus the difference is not confined to neonatal erythrocytes but, rather, reflects a genetic difference in the timing of the expression of H-2 antigens in the process of erythropoietic differentiation. A genetic analysis of this difference might thus provide important information on programmed gene action in mammalian cells (Stimpfling and Snell, 1962; Herberman and Stetson, 1965; Hilgert and Kristofová, 1971; Boubelík and Lengerová, unpublished).

MATERIALS AND METHODS

Mice. Mice used in these experiments were from the following inbred and congenic strains and some of their crosses bred at the Institute: A/P, C57BL/10ScSnP (symbol B10), B10.A/P, C57BL/6P (symbol B6), A.BY/P, B10.D2/P, HTG/P, B10.A(2R)/P, B10.A(5R)/P, C3H/P, B10.Br/P, AKR/P, B10.AKM/P, B10.C3H/NB/P, DBA/1/P, B10.RIII/P, B10.ASW/P (the substrain symbol P is omitted hereafter in this paper). Mice of the C57BL/6 TL^+ and A/TL^- strains were generously provided by Dr. E. A. Boyse, Sloan–Kettering Cancer Institute, New York.

Reagents. Reagents for the tests were prepared by a reciprocal immunization of B10 and B10.A strain mice. The immunization scheme closely followed that given by Snell (1968). For the sake of brevity, we refer to the antigens determined by $H\text{-}2^a$ as *a* antigens and by $H\text{-}2^b$ as *b* antigens. For detecting the antigen H-2.5 (one of a few antigenic specificities occurring in both *a* and *b* antigenic complexes), a specific antiserum corresponding to C-5 (Snell, 1968) was prepared by immunizing (B10.D2 × A.CA)F_1 mice with B10.A(5R) cells.

Erythrocytes. Red cells were tested for agglutinability on days 1, 2, and 3 after birth. Blood was obtained either by decapi-

tation or from the sigmoid sinus of the orbital branch of the anterior facial vein; a gentle puncture could be repeated daily alternating the left and right side. The amount of blood obtained from one puncture yielded an average of 0.2 ml of a 2.5% suspension of erythrocytes.

Haemagglutination. This was performed in the PVP (polyvinylpyrollidone) medium (Rubinstein and Kaliss, 1974). Since pooling of red cells from several individuals had to be avoided in some experiments and the amounts obtainable from a newborn donor (to be used repeatedly) were small, all the reactions were carried out with half of the usual volume of reagents and red cell suspensions. When the amount of red cells from a particular individual was very limited, the reaction was carried out only within the range of the optimal dilution of the respective reagent, as a rule between 1:20 and 1:320.

Classification of the "early" (E) type of either *a* or *b* was based on a demonstrated agglutinability by the specific H-2 reagent on the day of birth. By "late" (L) agglutinability we mean that a positive reaction occurred for the first time only on postnatal day 3. Table 1 illustrates that the difference between early and late agglutinability is clearcut so that the classification creates no problem. All *b* individuals neonatally appearing as L were retested when adult because *b* antigens agglutinate poorly. Those with an occasional poor agglutinability were discarded, because poor agglutinability might neonatally simulate lateness.

Analysis by selection and inbreeding. Starting from five pairs of H-2 homozygous (A × B10)F_2 individuals selected for reversed parental-strain timing types, five new inbred lines were established. Each generation the pairs were chosen not only for reversed timing but also for the easily detectable markers at the albino and brown loci in combinations as different as possible. (The complement levels of these mice were also measured.) Each line was then maintained by brother–sister mating and selection was continued in every generation for reversed parental-strain timing types.

RESULTS AND DISCUSSION

The results of typing a number of inbred and congenic strains for their H-2 timing are summarized in Table 2. Some of these data were reported previously (Boubelík and Lengerová, 1971). The following observations can be derived from these data.

1) Although the tested strains are of 11 different H-2 types, the timing types fall in merely two classes: E and L.

2) Late agglutinability is not correlated with a smaller number of H-2 specificities detectable by a given reagent; neither is it confined to particular H-2 specificities. This is best illustrated by the fact that the same specificity (such as H-2.5) or groups of specificities (such as $H\text{-}2^a$ products) may exhibit either early or late expression.

TABLE 1

ILLUSTRATION OF THE PRINCIPLE ON WHICH AGGLUTINABILITY OF ERYTHROCYTES IS CLASSIFIED AS EARLY/(E) OR LATE (L)

H-2 type, Classification	Strain	Agglutinability (log_2 titers) with H-2 reagents on stated postnatal days					
		$H\text{-}2^a$			$H\text{-}2^b$		
		Day 1	2	3	1	2	3
aL	A	–	–	8	—	–	—
bE	B10	–	—	—	6	7	8
bE	A.BY	–	—	—	6	8	8
aE	B10.A	7	7	8	—	–	—
aL/bE	(A×B10)F_1	–	–	8	6	6	7

TABLE 2

EARLY (E) VERSUS LATE (L) EXPRESSION OF H-2 ANTIGENS ON NEONATAL ERYTHROCYTES OF VARIOUS INBRED AND CONGENIC STRAINS AND H-2 SPECIFICITIES PUTATIVELY DETECTABLE BY THE ANTI-A AND ANTI-B REAGENTS USED

Strain		H-2 type	Timing of expression of H-2 specificities detectable by														
Symbol	Origin		Anti-a									Expression	Anti-b		Expression	Anti 5	Expression
			1	3	4	8	10	11	13	23	25		2	33			
A	Inbred	a	+	+	+	+	+	+	+	+	+	L				+	L
B10		b											+	+	E	+	E
B6		b											+	+	E	+	E
C3H		k	+	+		+		+		+	+	L				+	
AKR		k	+	+		+		+		+	+	E				+	
DBA/1		q	+	+				+	+			E					
B10.A	Congenic	a	+	+	+	+	+	+	+	+	+	E				+	E
A.BY		b											+	+	E	+	E
B10.D2		d		+	+	+	+		+			E					
B10.BR		k	+	+		+		+		+	+	E				+	
B10.AKM		m	+	+		+		+	+	+	+	E				+	
B10.C3H.NB		p	+	+		+						L				+	
B10.RIII		r	+	+		+	+				+	L				+	
B10.ASW		s	+	+								E				+	
B10.A(2R)	a-b Recombinant	h	+	+		+		+		+	+	E	+		E	+	E
B10.A(5R)		i		+	+				+			L		+	?	+	?
B6-T1a[a]		b											+	+	L	+	
A-T1a[b]		a	+	+	+	+	+	+	+	+	+	E				+	
A × B10	F_1 hybrid	a/b	+	+	+	+	+	+	+	+	+	L	+	+	E	+	E

3) Different timing phenotypes of strains of similar H-2 types (such as A and B10.A where the source of the *H-2*[a] chromosome is known to be the same) clearly suggest that a gene (or genes) other than *H-2* itself determines the timing type.

4) In H-2 heterozygotes, the timing types are expressed codominantly, i.e., *H-2*[b] *early* (bE) and *H-2*[a] *late* (aL). This fact, that the same cells display both early and late agglutinability depending on the H-2 reagent used, is a particularly strong argument in favor of the view that the difference in time of agglutinability reflects the developmental stage of H-2 antigens on the cell membrane rather than the development of the membrane or cell in general. The maintenance of parental timing types in H-2 heterozygotes further indicates that the timing gene (or genes) is operatively dependent in one way or another on chromosomal juxtaposition.

On the basis of these observations, we proceeded to analyze various segregating generations of crosses between contrasting strains. In the (A × B10)F_2 generation, in addition to the parental and F_1 phenotypes, new associations of H-2 and timing types were observed, namely aE and bL. Also in both reciprocal backcrosses, the aE type was found with certain frequencies.

The first tested but eventually rejected model was that consisting of two temporal loci linked to *H-2* and requiring the alleles determining late development to be concomitantly present at both loci on the same chromosome as the *H-2* allele for late expression of that *H* allele. This model is short lived for a number of reasons. First, in the backcrosses three individuals of the

phenotype aE/bE were found which should not occur in this model. Furthermore, in segregating generations from crosses of some other strains, only the parental and F_1 but no recombinant types were found (Table 3). In fact, there were 165 offspring from these crosses in which no reversed phenotypes were observed.

In addition to this observation, the number of generations taken to fix the recombinant types genetically in the selection experiment (Table 4) is inconsistent with the above model. The hypothetical recessive genotype required for late appearing H-2 types should have been true breeding at the outset.

These observations led us to abandon the first model and to propose another. To be acceptable the hypothesis must incorporate determinant(s) with a *cis*-like effect, which is reversible after a selection period of as many as 9–11 generations, and the determinant(s) must not necessarily show segregation in some strain crosses.

The hypothesis we propose, the *remote-cis-effect* hypothesis, has the following constraints.

1) The H-2 types alone *intrinsically* determine the time of development, i.e., antigens determined by $H\text{-}2^a$ develop late and by $H\text{-}2^b$ develop early. By intrinsic we do not necessarily mean that it is the structural locus itself that controls its timing. It might also be another tightly linked gene.

2) A gene unlinked to *H-2*, herein called *Tem*, is capable of overriding the intrinsic timing of *H-2*; allele Tem^a can cause antigens determined by $H\text{-}2^b$ to develop late, i.e., to simulate the intrinsic $H\text{-}2^a$ control, and allele Tem^b can cause antigens determined by $H\text{-}2^a$ to develop early.

TABLE 4

SEGREGATION OF H-2 TIMING TYPES WITHIN FIVE LINES ORIGINATING IN THE (A × B10)F_2 GENERATION FROM H-2 HOMOZYGOTES THEN MAINTAINED BY BROTHER–SISTER MATING AND SELECTED FOR THE REVERSED PARENTAL-STRAIN TIMING TYPE, EITHER aE OR bL, EVERY GENERATION THEREAFTER

Strain[a]	aE_1		aE_2		aE_3		bL_1		bL_2	
Phenotype	E	L	E	L	E	L	E	L	E	L
Generation										
0	2	0	2	0	2	0	0	2	0	2
1	8	1	8	7	4	4	10	2	7	1
2	7	1	14	6	7	3	4	4	4	2
3	4	3	21	6	9	13	6	3	4	9
4	5	12	6	2	4	12	1	6	8	6
5	4	5	11	7	7	8	5	10	3	7
6	7	10	3	3	7	7	4	5	4	6
7	24	2	18	21	4	21	3	15	8	5
8	31	4	30	5	12	20	4	22	11	16
9	12	0	29	5	19	13	0	12	7	10
10	44	0	34	0	32	3	0	20	1	23
11	20	0	36	0	27	0	0	18	0	10
12			26	0	35	0	0	13	0	17
13									0	15
14									0	19

[a] Meaning of symbols: aE_1 = *$H\text{-}2^a$ early*, albino, low complement level; aE_2 = *$H\text{-}2^a$ early*, black, high complement level; aE_3 = *$H\text{-}2^a$ early*, brown high complement level; bL_1 = *$H\text{-}2^b$ late*, black, ? complement level; bL_2 = *$H\text{-}2^b$ late*, brown, low complement level.

TABLE 3

PHENOTYPIC FREQUENCIES IN CROSSES OF OTHER bE AND aL STRAINS

Cross	Phenotypic classes								
	aL	aE	aL bE	aL bL	aE bE	aE bL	bE	bL	Total
[A.BY × A]F_2	18	—	42	—	—	—	17	—	77
[B10 × B10.A(5R)]F_2	9	—	18	—	—	—	6	—	33
B10.A(5R) × [B10 × B10.A(5R)]	23	—	32	—	—	—	—	—	55
Total									165

3) The presence of a gene linked to *Tem*, herein called *Rec*, is necessary and in the appropriate *cis* arrangement with *Tem* for *Tem* to be effective. It evokes the recognition of a specific H-2 type before the H-2 type can be affected by *Tem*. Allele Rec^a recognizes $H\text{-}2^a$ and allele Rec^b recognizes $H\text{-}2^b$. If, for example, Tem^b is not on the same chromosome with Rec^a but rather with Rec^b, then the intrinsic timing of $H\text{-}2^a$ expression will not be overridden and thus will be late, aL.

4) Accredited *Tem* alleles (those with a *Rec* allele that match the H-2 haplotype) are dominant over nonaccredited *Tem* alleles, e.g., ($Tem^a\ Rec^b\ H\text{-}2^b$)/($Tem^b\ Rec^a\ H\text{-}2^b$) is late, bL.

5) Accredited Tem^b (early) is dominant over accredited Tem^a (late), e.g., ($Tem^b\ Rec^a\ H\text{-}2^a/Tem^a\ Rec^a\ H\text{-}2^a$) is early, aE.

6) *Tem–Rec* complexes of either maternal or paternal source can control H-2 types of either maternal or paternal source, e.g., the genotype $Tem^a\ Rec^a\ H\text{-}2^a/Tem^b\ Rec^a\ H\text{-}2^b$, produced from an intrinsically aL father and an intrinsically bE mother, will have the phenotype of aE and bL. In this situation, the remote-*cis*-effect loses its resemblance to the conventional *cis* effect.

This model can satisfactorily accomodate all the experimental data on which it is based. Moreover, it is accessible to further tests. Table 5 gives the F_2 and backcross data with the phenotypic frequencies expected on the remote-*cis*-effect model. On first view, the overall fit given by the sum of the three χ^2 values is not good enough; the value 28.47 (13 *df*) exceeds the $P = 0.01$ level. However, the greatest contributions to χ^2 come from the backcross (A × B10) × A in which the expected frequencies of three out of five phenotypic classes are less than five. This condition does not meet one of the criteria for a valid χ^2 test. When these three classes are pooled to increase the expected frequency, χ^2 for this backcross reduces to 0.92 (2 *df*), which is not significant with $P > 0.70$; and in the over-all fit, χ^2 reduces to 11.78 (11 *df*), which is not significant with $P > 0.30$. It also should be noted that the occurrence of unexpected phenotypes, a basic shortcoming of the first model tested, is abolished in this model.

A further advantage of the remote-*cis*-effect model is that it explains the absence of recombinant H-2 timing types in the crosses listed in Table 3. Table 6 gives a complete list of genotypes based on all possible combinations of alleles *Tem* (*a*,*b*), *Rec* (*a*,*b*) and *H-2* (*a*,*b*) with appropriate phenotypes. Table 7 then presents hypothetical genotypes that explain the phenotypes (and the lack of segregation in the appropriate crosses) of some of the strains from Table 2 and also genotypes of the new lines obtained by inbreeding and selection.

TABLE 5

FREQUENCIES OF H-2 AND TIMING PHENOTYPES AS EXPECTED AND OBSERVED IN THREE DIFFERENT SEGREGATION GENERATIONS OF THE CROSS (A × B10) ON THE REMOTE-*cis*-EFFECT MODEL WITH THE DISTANCE OF 20 CM BETWEEN *Tem* AND *Rec* LOCI[a]

Cross		Phenotypic classes									χ^2 (*df*) *P*
		aL	aE	aL bE	aL bL	aE bE	aE bL	bE	bL	Total	
(A × B10)F_2	Observed	35	16	82	6	18	0	52	4	213	9.83 (7)
	Expected	43.13	10.12	76.68	9.58	18.11	2.13	47.39	5.86		P > 0.10
(A × B10) × A	Observed	40	13	42	3	1	0	0	0	99	17.62 (4)
	Expected	44.55	4.95	39.60	4.95	4.95	—	—	—		P < 0.001
(A × B10) × B10	Observed	0	0	36	0	2	0	41	0	79	1.03 (2)
	Expected	—	—	35.55	—	3.95	—	39.50	—		P > 0.50

[a] The distance is a mean value of the estimates from all three sets of data.

TABLE 6

GENOTYPES BASED ON ALL POSSIBLE COMBINATIONS OF ALLELES *Tem* (A, B), *Rec* (A, B) AND *H-2* (A, B) WITH THE APPROPRIATE TIMING PHENOTYPES (E OR L)[a]

H-2	Timing phenotype for the stated temporal complex genotype															
	$\frac{aa}{aa}$	$\frac{ab}{ab}$	$\frac{aa}{ab}$	$\frac{ab}{aa}$	$\frac{ba}{ba}$	$\frac{bb}{bb}$	$\frac{ba}{bb}$	$\frac{bb}{ba}$	$\frac{aa}{ba}$	$\frac{ab}{bb}$	$\frac{aa}{bb}$	$\frac{ab}{ba}$	$\frac{ba}{aa}$	$\frac{bb}{ab}$	$\frac{ba}{ab}$	$\frac{bb}{aa}$
a	L	(L)	L	L	E	(L)	E	E	E	(L)	L	E	E	(L)	E	L
b	(E)	L	L	L	(E)	E	E	E	(E)	E	E	L	(E)	E	L	E

[a] E, early; L, late; ( ), intrinsic determination; allele (*a* or *b*) at *Tem* locus given first and at *Rec* locus second, e.g.,

$$\frac{Tem^a\ Rec^a}{Tem^b\ Rec^a} = \frac{aa}{ba}$$

TABLE 7

HYPOTHETICAL TEMPORAL-COMPLEX GENOTYPES WHICH CAN EXPLAIN KNOWN H-2 TIMING PHENOTYPES OF SOME INBRED STRAINS

Strain	Alleles at the stated loci		Phenotype
	Tem Rec	*H-2*	
B10	$\frac{bb}{bb}$	$\frac{b}{b}$	bE
A	$\frac{aa}{aa}$	$\frac{a}{a}$	aL
B10.A	$\frac{ba}{ba}$	$\frac{a}{a}$	aE
A.BY	$\frac{aa}{aa}$	$\frac{b}{b}$	bE
bL (ex selection)	$\frac{ab}{ab}$	$\frac{b}{b}$	bL
aE (ex selection)	$\frac{ba}{ba}$	$\frac{a}{a}$	aE

Finally, Table 8 includes expected and observed results of several crosses which were suggested for testing the model independently, i.e., on the basis of experiments which were not used for its construction.

With regard to the possible determinant of the postulated "intrinsic" timing, inbred congenic lines that carry recombinant chromosomes might be of great value. The timing types of congenic strains, B10.A(2R) and B10.A(5R) (Table 1), whose *H-2* chromosomes have arisen through reciprocal recombination of *H-2*^{a} and *H-2*^{b} within the *H-2* gene complex, suggest that a temporal gene might be linked with the D end of the *H-2* complex, proximal to the *Tla* locus. If this were the case, crossing over between *H-2* and *Tla* might possibly result in reversal of the timing types. The two H-2 recombinant congenic lines A-*Tla*^{b} (TL$^-$) and B6-*Tla*^{a} (TL$^+$) carry chromosomes of just this type, i.e., reciprocal crossovers between *Tla* and the D end of *H-2*. The progenitor strains, A and C57BL/6 (B6) are respectively aL, TL$^+$, and bE, TL$^-$. The recombinant chromosomes are associated with reversed H-2 timing types: the B6-*Tla*^{a} strain is bL, TL$^+$ and the A-*Tla*^{b} strain is aE, TL$^-$. On the basis of this observation the "intrinsic" timing might be controlled by a temporal gene linked to the D end of *H-2*, which was separated from *H-2* together with *Tla*. Thus, the complete "temporal" genotypes (including the "intrinsic" temporal locus *Int*) of the two strains would be as follows:

$$\text{A-}Tla^b\text{:}\quad \frac{Tem^a\ Rec^a}{Tem^a\ Rec^a}\ \frac{Int^b\ H\text{-}2^a}{Int^b\ H\text{-}2^a};$$

$$\text{B6-}Tla^a\text{:}\quad \frac{Tem^b\ Rec^b}{Tem^b\ Rec^b}\ \frac{Int^a\ H\text{-}2^b}{Int^a\ H\text{-}2^b}.$$

A pertinent question concerns the possible mechanism through which the postulated temporal genes might operate. Muta

TABLE 8

FREQUENCIES OF H-2 AND TIMING PHENOTYPES AS OBSERVED IN INDEPENDENT EXPERIMENTS AND AS PREDICTED FROM THE REMOTE-*cis*-EFFECT MODEL AND THE PERTINENT GENOTYPES IN TABLE 7

Strain cross		Phenotypic classes						χ^2	(*df*) *P*
		aE	aL	aL bE	aE bE	bE	Total		
(B10.A × A.BY)F_2	Expected	25.875	8.625	17.25	51.75	34.5		4.9	(4)
	Observed	30	6	10	55	37	138		$P > 0.30$
(B10.A × A.BY) × B10.A	Expected	22.5			22.5			0.1	(1)
	Observed	24			21		45		$P > 0.70$
(B10.A × A.BY) × A.BY	Expected			7	7	14		0.28	(2)
	Observed			8	7	13	28		$P > 0.80$
(B10.A × A)F_2	Expected	26.25	8.75					0.6	(1)
	Observed	27	8				35		$P > 0.80$
(B10.A × A) × A	Expected	35	35					0.49	(1)
	Observed	38	32				70		$P > 0.50$
(B10.A × A) × B10.A	Expected	54							
	Observed	54					54		

ions of temporal genes, which might elucidate the normal mechanism, would probably tend to misprogram developmental processes, be consequently lethal and be selected against. A very illuminating example of drastic developmental defects due to a mere misprogramming of a process of embryonic induction seems to be provided by the mutation *Sd* in mice; as demonstrated by *in vitro* experiments, both the inducing and induced tissue components are normal, but they fail to make the proper contact at the right time under conditions *in vivo* (Gluecksohn-Waelsch, 1965).

With our present state of knowledge, developmental timing can be explained most simply on the basis of a series of sequentially dependent activations of genes. Each such gene in turn could assist in activating by its product either inter- or intracellularly the next gene in sequence. Timing of any one particular event could be realized through rates of the preceding sequence of synthesis, intracellular assembly, degradation, transport, secretion, diffusion, and uptake of activating (induction-like) substances and competence-determining substances. *Any* preceding gene in the sequence could be considered a *temporal* gene.

In the present instance, the hypothetical "temporal complex" contains a gene that supposedly determines when H-2 antigens are attached to the membrane. *Rec* has been described as a specific allele-(haplotype)-recognizing gene, however the level of recognition could be anywhere from that of the gene to the gene product. *Rec* might well be a structural gene that produces a membrane-bound macromolecule which facilitates the attachment of the H-2 product and which recognizes the product of $H\text{-}2^a$ differently from that of $H\text{-}2^b$. Perhaps *Tem* determines the rate at which *Rec* synthesizes its product.

Genes that modify the development of cell-surface antigens have been described for other systems. The *y* locus, unlinked to the ABO locus in man was inferred by Weiner *et al.* (1957) from data on two families to determine the development of antigen A but not of antigens B or H on red blood cells. Similarly, a gene unlinked to HL-A in man appeared to have control over the expression of antigen W29-W10 (Bias *et al.*, 1974). The modifiers in these two systems resemble one of those we hypothesize here in their being unlinked to the structural locus and in their showing specific antigen modification, that is, the product of one allele but not the other was

altered. However not enough data are available on those systems to carry the parallelism further.

The model presented here resembles somewhat the systems found by McClintock (1967) to control pigment in maize. Those systems consist of two "controlling elements," one of which is unlinked (as our temporal complex) and one tightly linked to the structural gene (as our intrinsic timing control at the D end of the *H-2* complex). It becomes fruitless, however, to pursue this comparison further without more supportive data on the H-2 timing system.

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BRIEF NOTES

A Biological Nuclear Marker in Cell Culture: Recognition of Nuclei in Single Cells and in Heterokaryons[1]

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The biological marking technique based on quail and chick cell combinations, previously devised for *in vivo* experiments and organotypic cultures, can also be applied to cell cultures. In the experiments reported in this note, quail and chick fibroblasts and cardiac cells have been cultivated from 2–8 days. Up to the end of the second day of cultivation, the nucleolar structure of quail cells remains similar to that observed in organized tissues. Afterwards, the nucleolar enlargement which occurs in rapidly growing monolayer cultures results in the fragmentation of the characteristic nucleolus-associated heterochromatin in quail nuclei. However, since chick nuclei show only small dispersed chromocenters, cells of the two species can be identified even after several days in culture. Acridine orange staining is shown to be an easy and reliable procedure for identifying the nuclear marker, even in heterokaryons. Cytoplasm stains to distinguish cell types can also be applied to the same preparations.

INTRODUCTION

A biological cell-marking technique based on structural differences of the nucleus in two species of birds, the Japanese quail (*Coturnix coturnix japonica*) and the chick (*Gallus gallus*), has been previously described by Le Douarin (1969, 1971, 1973a, b). This technique has been used successfully in combination experiments, either in organotypic culture or by grafting quail rudiments into chick embryo (Le Douarin and Jotereau, 1973; Le Douarin and Le Lièvre, 1970; Le Douarin *et al.*, 1972; Le Douarin and Teillet, 1973, 1974). Under such conditions the cells of the two species retain their nuclear characteristics in the chimaeras and can be identified regardless of the duration of the tissue association. In quail cells one or several masses of heterochromatic DNA are associated with the nucleolus in all embryonic and adult cell types. In the chick such a characteristic does not exist and the amount of heterochromatic DNA and nucleolus-associated chromatin is small. As a result of this characteristic of quail nuclei, the distinction between quail and chick tissues *in situ* is easy to make in light microscopy after appropriate staining with the Feulgen–Rossenbeck (1924) procedure (Fig. 1).

The experiments reported here were designed to see whether the quail–chick marking technique could be used in cell cultures. The questions asked were 1) is the quail nuclear feature maintained under cell culture conditions and 2) could a simple staining procedure be used to distinguish the cells of the two species? If the nuclear marker technique also applies to cell cultures with appropriate staining, would these procedures lend themselves to the analysis of cell fusion experiments? The possibility of being able to distinguish heterokaryons from homokaryons could be of interest when the fusing cells do not show specific morphological distinguishing characters.

In this brief paper we report that the

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questions posed can be answered positively.

MATERIALS AND METHODS

White Leghorn chick and Japanese quail eggs, incubated at 38 ± 1°C were used throughout this investigation. Two kinds of cultures have been carried out: (1) Fibroblasts from eviscerated trunks of 8-day-old quail and chick embryos; 2) cardiomyoblasts taken at 7 days of incubation from the two species.

Cultures. Eviscerated trunks were fragmented in small pieces and the tissues were incubated 30 min at 38°C in a CA^{2+}-Mg^{2+} free Tyrode's solution containing 0.25% trypsin (Nutritional Biochemicals Corporation, Cleveland). The resulting cell suspension was filtered through sterile gauze and the filtrate thoroughly washed with four changes of Eagle's medium (MEM) supplemented with 10% fetal calf serum and antibiotics (penicillin, 100 units/ml; streptomycin, 100 μg/ml). The cells were dispersed in the same medium by gentle flushing with a 0.7-mm Pasteur pipet. Two milliliters of the suspension at a concentration of 1×10^6 cells/ml were distributed into petri dishes (35 × 10 mm) containing a coverslip. Cultures were incubated at 37.5°C for 24 hr–8 days in an incubator with humidified atmosphere composed of 95% air and 5% CO_2.

For cultures of cardiac cells, a concentration of trypsin (0.05% in Ca^{2+}-, Mg^{2+}-free Tyrode's) lower than for fibroblasts was used (Renaud and Le Douarin, 1972).

Cell fusion. Cells were fused after 24 hr in culture using uv-inactivated Sendai virus (1000 haemagglutinating units/ml). Quail and chick cells were cultivated at a total concentration of 2×10^6 cells/ml (1×10^6 quail + 1×10^6 chick cells) in the same petri dish, the total amount of medium per culture being 2 ml. Cultures were first rinsed with cold serum-free culture medium and then incubated for 10 min at 4°C and 20 min at 37°C in Eagle's medium containing the inactivated virus. The nonabsorbed viruses were removed by rinsing with medium, and the cells were then cultivated according to the procedures mentioned above for 24 hr.

Cytological techniques. Cells attached on coverslips were fixed 10 min in Carnoy fluid and treated either according to classical Feulgen–Rossenbeck procedure for DNA or stained with acridine orange. In the latter case, after fixation, the coverslips were rinsed in McIlvaine buffer (pH 5–6) and immersed in a solution of acridine orange for 2 min. After several rinses with the buffer, the cells were observed in uv light using a Leitz Orthoplan microscope fitted with an HBO 200 W mercury arc lamp. Filters used were BG 12/5 mm, BG 12/3 mm, BG 12/1.5 mm for excitation with a K 510 barrier filter. Photomicrographs were taken on high-speed ektachrome EHB 135-20 Kodak film (ASA 200).

In some cases cardiac cells, first stained with acridine orange and observed in uv light, were posttreated (Hotchkiss, 1948) for detection of polysaccharides. The glycogen nature of the polysaccharides was tested with amylase digestion prior to application of staining procedures.

RESULTS

Cultures of Fibroblasts

After 24–48 hr in culture quail cells fixed on the coverslip show the same large dense nucleolus observed in embryonic (Fig. 1) and adult tissues. In phase contrast microscopy quail and chick cells can in most cases be easily distinguished. The nucleolar heterochromatic DNA in the quail nucleus appears as a prominent dark spot, while the RNA components of the nucleolus are much lighter in appearance. When the cells are treated by the Feulgen–Rossenbeck procedure, the characteristics of quail and chick nuclei are the same as in the intact embryo prior to transplantation. In cells stained with acridine orange, the differences between the two species are particularly conspicuous (Figs. 2 and 3). In

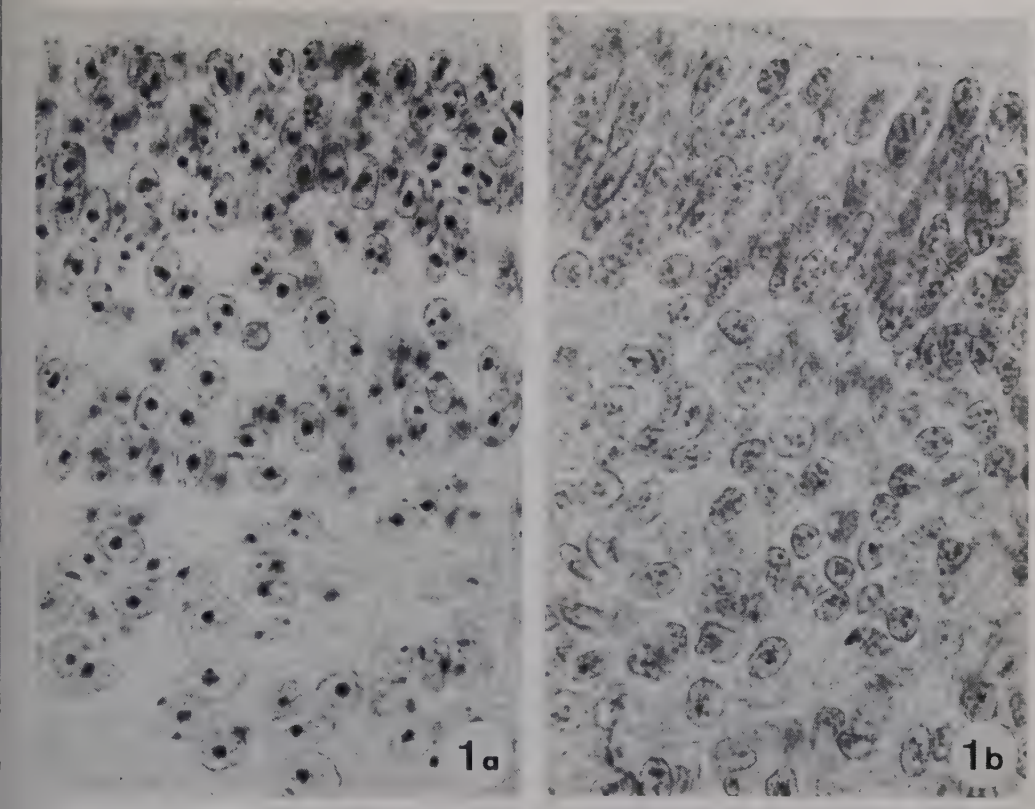


FIG. 1. Nuclear differences between quail (a) and chick (b) trunk cells (spinal cord neuroblasts) after Feulgen Rossenbeck staining. Section of the trunk of 8-day-old embryos before transplantation. The same feature is observed in the nucleus of all kinds of trunk cells: Large heterochromatic mass associated with the nucleolus in the quail, evenly distributed chromatin in the chick with small scattered chromocenters. × 1000.

quail cells, the nucleus shows one or several bright yellow-greenish masses of heterochromatin associated with the red-stained nucleolar RNA. The relationships between RNA and DNA components are essentially those described by electron microscopy in type 1 nucleoli of quail; RNA is located beside the heterochromatin in one, two or three masses (Le Douarin, 1973b). Fluorescence of euchromatic DNA distributed in the nucleoplasm is much fainter than that of condensed nucleolar chromatin. In chick cells, the nucleus shows a yellow-greenish fluorescence with small scattered chromocenters. The red-stained nucleolus is surrounded by a thin ring of yellow-green DNA slightly brighter than the nucleoplasm; some cells show small perinucleolar chromocenters.

After 2 days in culture, the nucleolus of most quail cells still shows one or two compact masses of brightly fluorescent heterochromatic DNA. The amount of nucleolar RNA is increased. In some cells the heterochromatin is fragmented in several masses located around or inside the red-stained RNA component of the nucleolus. The number of cells showing nucleolar modifications of these kinds is at about 20% in 3-day cultures, 50% at 4 days and 80% at 5 days, until the end of the culture period which has been prolonged to 8 days in some experiments. In chick cells after 8 days *in vitro*, the nucleolus appears as a large red spot in the rather uniform yellow-greenish fluorescence of the dispersed nuclear chromatin.

In cell fusion experiments, the cells are

observed after 48 hr in culture. The homokaryons of quail or chick cells containing, respectively, two quail or two chick nuclei (Fig. 4) are readily identified by their nucleoli. The heterokaryons resulting from the fusion of chick and quail cells are easily distinguished by their nucleolar characteristics.

Cultures of Cardiac Cells

After 24 hr in culture, 60–70% of the cells attached to the coverslip are spherical and most of them are pulsatile. The others stretched on the substrate are fibroblastic in appearance and only rarely do these cells show pulsation. When stained with acridine orange the cells of the two species show the same conspicuous differences in the features of their nuclei noted above. In spherical cells of quail, the nucleus contains a single bright heterochromatic mass usually associated with several patches of nucleolar RNA. In the quail fibroblast-type cell, two or three nucleoli are often encountered.

The nuclear structure is the same in spherical and fibroblastic chick cells. They show a red-stained nucleolus surrounded by a thin ring of DNA, usually associated with fluorescent chromocenters. After action of Sendai virus in mixed cultures, heterokaryons and homokaryons are observed. Numerous pulsatile binucleated cells of the spherical type can be recognized as soon as 2 hr after the addition of the virus to the culture. In phase contrast microscopy, the large DNA-rich nucleolus of quail origin can be in most cases readily identified. Sometimes however, the observation of the nucleus is difficult due to the presence of refractile cytoplasmic inclusions. After staining with acridine orange the identification of the nuclei of the two species is easy in normal and binucleated cells (Fig. 5). Cultures of cardiomyoblasts have not been prolonged more than 48 hr in our experiments.

After staining with acridine orange, a glycogen stain was applied in some cases. The characteristic staining of cardiac myocytes following the PAS reaction (Hotchkiss, 1948) was observed and the glycogen nature confirmed by the amylase digestion test.

DISCUSSION

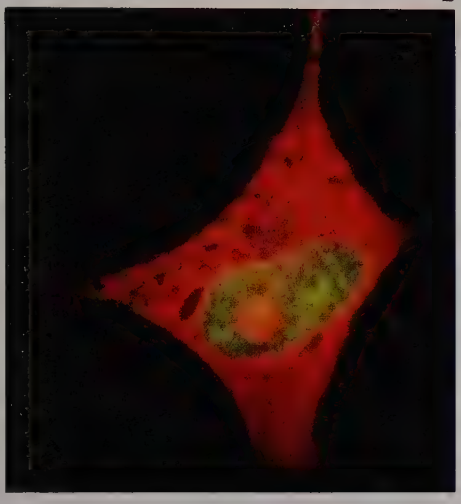
Quail and chick fibroblasts and cardiac cells growing in cell culture can be recognized by specific nuclear features. During the first 2 days of cultivation, the nucleolus of quail cells shows a structure similar to that observed in organized tissues from embryonic and adult quail, that is, large masses of nucleolar heterochromatic DNA. Subsequently, the nucleolus enlarges and the nucleolar substructure becomes modified. The large heterochromatic mass that characterizes the quail nucleolus becomes fragmented into several smaller patches. In chick cells, the nucleolus is mainly made up of RNA and, as is usually the case in other animal species, the amount of nucleolus-associated chromatin is small (Bernhard and Granboulan, 1968). In chick cell cultures the activation of growth and protein synthesis results in nucleolar enlargement with no change in structure which can be detected at the light micro-

FIG. 2. Chick cell from eviscerated trunk, cultivated 24 hr, stained with acridine orange. Red-stained nucleolus made up mainly of RNA with a small amount of associated chromatin × 900.

FIG. 3. Quail cell from eviscerated trunk, cultivated 48 hr. A large DNA heterochromatic clump showing a bright yellow fluorescence is associated with the red-stained nucleolar RNA × 900.

FIG. 4. Action of inactivated Sendai virus on a mixed culture of quail and chick fibroblasts: Homokaryon resulting from the fusion of two chick cells. × 900.

FIG. 5. Heterokaryon following fusion of quail and chick cardiomyoblasts. The quail nucleus (below) exhibits large yellow DNA masses associated with the nucleolus, while in the chick nucleus the red-stained nucleolar RNA is surrounded by small chromocenters. The latter have often been found associated with the nucleolus in cardiac cells. In fibroblast cultures, on the contrary, the nucleolus-associated DNA appears most often as a thin yellow ring around the RNA component. × 1700.



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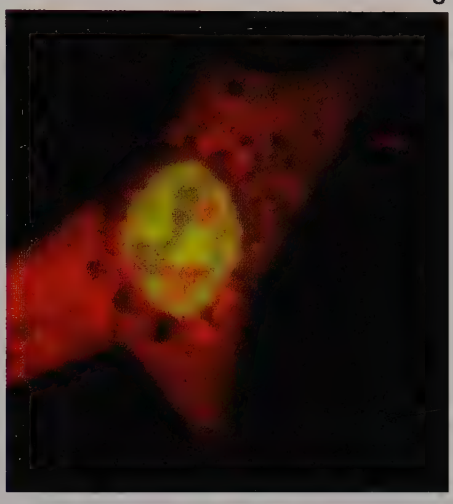

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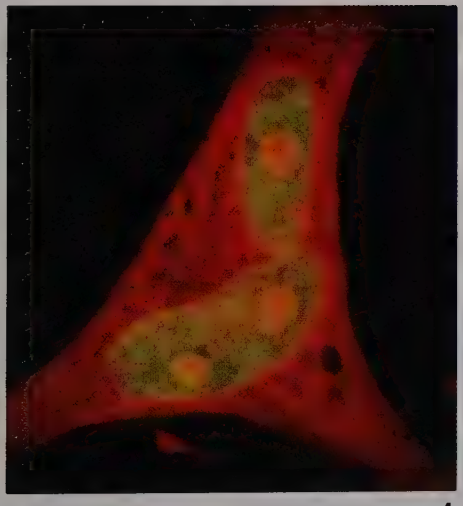

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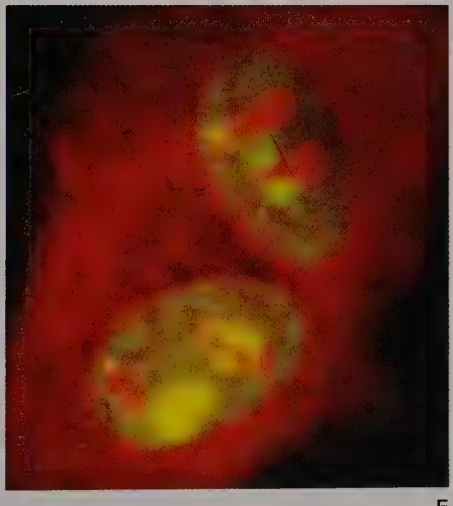

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scope level. Thus, even when the nucleolus-associated DNA became fragmented in quail cells after some days in culture, quail and chick nuclei can be recognized. In chick cells, the numerous and conspicuous chromatic condensations observed in the quail nucleolus are never present.

In vivo observation of cells with a phase contrast microscope makes it possible, in about 50% of the cases, to distinguish quail from chick cells during the first days of culture. With Feulgen–Rossenbeck or acridine orange staining, however, reliable distinction can be made among all the cells. With acridine orange, which is easier to apply than the Feulgen–Rossenbeck staining procedure, heterochromatic DNA of the quail nucleus appears as a brightly fluorescent yellow-green spot associated with the red-stained nucleolar RNA. In chick cells, the nucleoplasm shows a uniform yellow-greenish fluorescence in which the nucleolus stands out in red; only a few small chromocenters appear brighter than the background fluorescence of the nucleus.

Cytochemical reactions, such as the PAS staining procedure, can be applied to cells first stained with acridine orange and observed in uv light. Thus it is possible to determine for each nucleus whether it belongs to the quail or chick species and whether or not the cytoplasm has the high polysaccharide content typical of myocytes.

This cell-recognition technique based on nucleolar features can be applied in cell fusion experiments to recognize homo- and heterokaryons. Due to the fact that all the cell types of quail show the nuclear marker, such a distinction can be made whatever the state of differentiation of the cultured cells may be.

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DEVELOPMENTAL BIOLOGY 47, 222–227 (1975)

Clonal Analysis of Vertebrate Myogenesis

V. Nerve–Muscle Interaction in Chick Limb Bud Chorio-Allantoic Membrane Grafts

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The clonable cell populations of innervated and noninnervated chorio-allantoic membrane (CAM) grafts of early chick embryo leg buds have been compared. Co-grafts of stage 21 or 22 leg buds with stage 22–26 spinal cord segments exhibit a near twofold greater proportion of clonable muscle cells than noninnervated control grafts (leg bud alone). A statistically significant increase is apparent by the 8th graft day. Grafts constructed of stage 17–20 leg buds plus spinal cord and grown for up to 11 days do not exhibit a proportion of myoblasts greater than noninnervated controls. This stimulation of relative muscle cell content suggests a role of motor nerves in regulation of the single-cell population of developing skeletal muscle.

INTRODUCTION

Clonal analysis of normal chick embryo muscle development has shown that clonable myoblasts first appear in the leg soft tissue during the 3rd day of egg incubation, stage 21 of Hamburger and Hamilton (1951). The fraction of clonable cells which differentiates into muscle in conditioned medium increases rapidly from zero at day 3 to a plateau of 55–60% by day 5; a second rapid rise to about 90% occurs between days 7.5 and 9. The value of 90% is maintained throughout the rest of embryonic development. In fresh medium the proportion of clonable myoblasts increases smoothly from zero on day 5 to about 35% by day 10 (Bonner and Hauschka, 1974, White *et al.*, 1975). In this paper an attempt is made to determine whether any of the changes undergone by the clonable myoblast population are nerve dependent.

To minimize effects of other tissues, interaction between muscle and motor nerve was allowed to occur in chorio-allantoic membrane (CAM) co-grafts of early leg bud and spinal cord. A clonal analysis of grafted tissues was performed to determine whether innervated grafts exhibited a greater proportion of clonable myoblasts than noninnervated control grafts.

In the normal chick embryo leg, the changes in the clonable myoblast population most likely to be influenced by innervation would be those occurring after days 6 and 7 because it is during this 2-day interval that nerve-dependent limb movement and sensitivity to neuromuscular blocking agents appear (Hamburger, 1968; Ahmed, 1967). The second rise in the proportion of clonable myoblasts, occurring between days 7.5 and 9, then, is the most likely candidate for a nerve-dependent change in the single-muscle-cell population. This change should be reflected by a sudden increase in clonable myoblast proportion in long-term innervated grafts. Such a temporally delayed increase has been observed to begin during the 8th graft day.

MATERIALS AND METHODS

Dissection and grafting procedure. All operations were performed using sterile technique. Donor white Leghorn embryos were placed in culture-medium-containing dishes and their leg buds removed by cutting along the flank line with fine scalpels.

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Spinal cord segments were obtained from a section of trunk taken from midway between the wing and leg to the posterior end of the leg. The trunk section was placed on its side and the gut cut away and discarded. From the dorsal side, cuts were made along either side of the spinal cord, as close to the cord as possible. The resulting spinal cord and notochord-containing piece was treated by trimming away all tissue ventral to the notochord, leaving spinal cord, notochord and intervening tissue. Cross cuts were then made resulting in segments equal in length to a stage 20–22 leg bud. If spinal cord alone was to be used for grafting, the notochord and intervening tissue was cut away. Donor tissues were kept in a 37°C, air–CO_2, humidified incubator until grafted to the CAMs of 8-day-old hosts prepared according to the procedure of Zwilling (1959).

Treatment of graft tissue. After 6–11 days of growth on the CAM, grafts were removed and placed in fresh medium-containing dishes. Remaining host membrane, as much graft skin as possible, and the graft toes and ankles were removed and discarded. Techniques used for reducing the graft soft tissue to a single-cell suspension and the conditions of clonal culture have been described previously (Bonner and Hauschka, 1974; White *et al.*, 1975).

Culture medium. Fresh medium is 79% Ham's F-10 nutrient solution, 1% penicillin–streptomycin (10,000 units of penicillin G, 0.5 mg/ml of streptomycin sulfate), 15% preselected horse serum, and 5% day-12 chick embryo extract (Konigsberg, 1968; Hauschka, 1972). Conditioned medium is fresh medium that has been exposed, in 20-ml aliquots, for 24 hr to confluent secondary cultures of chick leg muscle cells grown in 100-mm petri dishes (Falcon 3003).

RESULTS

Chick limb buds grown as CAM grafts approach normal morphology to varying degrees (Hunt, 1932; Bradley, 1970). Some leg grafts, after 6–10 days of growth, appear fairly normal in terms of bone structure, number of toes, scales, feather growth etc. Others appear as shapeless lumps of tissue containing a few unrecognizable bones. Most grafts, however, are of intermediate morphology and, in this series of experiments, tend toward the normal. In the present experiments grafts were of two types, stage 17–23 leg buds alone or leg buds grafted with a source of innervation (embryonic spinal cord of stage 22–26 with or without the attached subjacent notochord). Early in this work it was found that inclusion of the notochord, notochordal sheath and intervening tissue along with the short spinal cord segment allowed successful grafting of the neural portion more often than with spinal cord alone. For this reason most innervated grafts consisted of a leg bud next to a segment of spinal cord plus notochord (SCN). Of the total number of grafts attempted, approximately one-third were successful. Innervated and noninnervated grafts were indistinguishable in terms of size, morphology and cell number.

Particular grafts were selected for clonal analysis only if they were of near-normal morphology, exhibiting at least two toes, recognizable femur, tibia, and fibula, well-formed joints, pieces of pelvic girdle, and feathers on older grafts. Table 1 represents data collected from 52 such grafts. Nearly all of the innervated grafts exhibited muscle contraction beginning on about the 3rd graft day. This neuromuscular activity ranged from small, localized fasciculations to large displacement twitches of the entire muscle mass. Noninnervated grafts and those containing only notochord and leg bud were never observed to move. To determine whether innervation can affect myogenesis at the myoblast level, graft soft tissue was analyzed by clonal growth and differentiation of single cells in conditioned and fresh medium. Pictures of repre-

TABLE 1

CLONAL ANALYSIS OF INNERVATED AND NONINNERVATED LEG GRAFTS

	Graft combination[a]		Duration of graft (days)	Number of experiments	Percent muscle colony differentiation[b]	
					Conditioned medium	Fresh medium
Noninnervated	A	17–20 leg	6–11	11	23 ± 12	3 ± 1
	B	21–22 leg	6–7	4	26 ± 17	8 ± 5
	C	21–22 leg	8–10	10	28 ± 12	13 ± 9
Innervated	D	17–20 leg / 22–26 SCN	6–11	6	23 ± 12	—
	E	21–22 leg / 22–26 SCN	6–7	3	32 ± 10	9 ± 6
	F	21–22 leg / 22–26 SC or SCN	8–10	11	51 ± 8	29 ± 7
Notochord control	G	20–23 leg / 22–26 Noto	8–10	7	29 ± 15	—

[a] Expressed as stage of donor leg and nerve source at time of grafting. SCN, spinal cord plus notochord; SC, spinal cord; Noto, notochord.

[b] Percent of total clones that contain clearly discernible multinucleated myotubes ± standard deviation. Significant differences in percent muscle colony differentiation between innervated and noninnervated grafts exist only between rows C and F (in conditioned medium, $P < 0.001$; in fresh medium, $P < 0.01$). Row-F values are significantly different than row-E values (in both media, $P < 0.01$), row-D values (conditioned medium only, $P < 0.001$), and row-G values (conditioned medium only, $P < 0.01$). All P-values were determined according to Student's two-tailed t test. Plating efficiencies, expressed as (total clones ÷ total number of cells inoculated) × 100, in conditioned medium and fresh medium are: Row A, 10.7 ± 2.9 and 10.8 ± 1.7; row B, 6.0 ± 0.8 and 6.7 ± 3.1; row C, 8.8 ± 4.2 and 10.1 ± 3.0; row D, 9.9 ± 4.7 and 9.1 ± 5.7; row E, 12.2 ± 1.9 and 12.9 ± 2.5; row F, 11.1 ± 5.5 and 9.5 ± 2.8; row G, 8.2 ± 4.6 and 9.3 ± 4.7.

sentative clones are presented in Fig. 1.

The clonal analysis data of Table 1 suggest that motor innervation causes increased percentages of clonable myoblasts in grafted leg buds but this effect depends upon the age of the donor leg bud and upon the duration of the graft. By comparing rows A and D of the table it can be seen that leg buds grafted at stages 17–20 and grown for 6–11 days on the CAM do not respond to a co-grafted nerve source. Grafted stage 21 and 22 leg buds also do not respond by the 6th and 7th graft days (rows B and E), but by the 8th–10th graft days they do exhibit a statistically significant increase in the myoblast fraction of the clonable cell population as compared to noninnervated control grafts (rows C and F). The magnitude of the increase is essentially the same in both media. Comparison of rows C and G indicates that the notochord alone is incapable of eliciting a similar response. Four of the 11 row-F grafts were of the notochordless type "leg bud plus spinal cord alone" and gave an average percent muscle colony differentiation of 53 ± 3. Because of the lack of a notochord effect and because spinal cord alone allows a complete response, it is felt that the above twofold increase in myoblast content reflects a nerve-specific effect on myogenesis.

DISCUSSION

The CAM graft technique was selected for these initial studies of nerve–muscle interaction during development because the developing limb bud and nerve source can, except for vascular connection, be kept physically separate from the embryo.

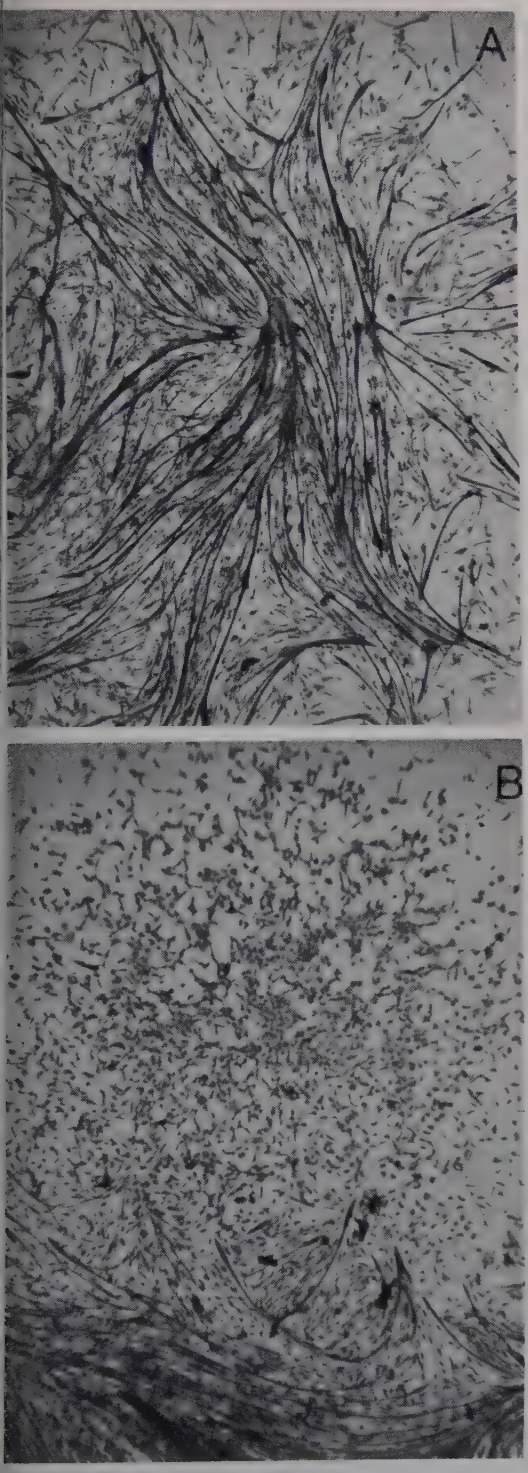

FIG. 1. Fixed and stained clones grown for 13 days in conditioned medium from cells derived from a 10-day-old CAM graft constructed of a stage 21 leg bud and a segment of stage 22 spinal cord. (A), A muscle clone; 12.5×. Note the many long, swirling, multinucleated myotubes. (B), A small, nonfusing clone immediately adjacent to a fused muscle clone; 12.5×.

The technique has limitations in that every graft is abnormal simply because of the separation. Neuromuscular connections were assumed to have occurred in innervated grafts because of the spontaneous muscle twitching observed; but the degree to which these connections are normal is unknown. Within these limitations, however, the data of Table 1 can still be useful for understanding more about the relationship of nerve to muscle development.

When early (stage 21–22), preinnervation leg buds are grafted with a motornerve source, the fraction of myoblasts in the clonable cell population remains the same as noninnervated control grafts through the 6th and 7th graft days. Innervated grafts taken for clonal analysis on the 8th–10th days produce a proportion of differentiated clones about twofold higher than noninnervated control grafts. This effect could be a result of nerve-dependent conversion of myogenic precursor cells into clonally identifiable myoblasts and/or selective proliferation of the myoblast population. The temporal delay in appearance of the effect is consistent with these possibilities. Alternatively, greater atrophy and degeneration of muscle tissue in noninnervated control grafts (Hunt, 1932; Eastlick, 1943) could result in a relative decrease of muscle cell content. While selective degeneration is a real possibility, it is unlikely to account for the entire innervation effect. Two parameters which should be affected by degeneration, gross morphology and single cell content, exhibit no change dependent on innervation during the course of these grafts. Further, if degeneration is involved, one might expect the percent muscle colony differentiation

to decrease with increasing age of noninnervated grafts. Such a decrease has not been observed.

Very early leg buds (stages 17–20), even when grown with a nerve source for up to 11 days on the CAM, do not contain a greater proportion of myoblasts in the clonable cell population than do controls. This dependence on developmental age of the donor leg bud is mysterious since the grafts are similar in all other respects to those made from the slightly older stage 21–22 buds. Perhaps the cells in the very young buds do not respond because of the greater disparity in age between the leg bud and the grafted spinal cord. Another possibility is that these very young buds are incapable of carrying out some process required for subsequent response to innervation when grown as grafts, a process which the 1-day-older stage 21–22 buds have completed before being removed from the embryo. Another interesting facet of these stage 17–20 grafts is that, at the time of grafting, the leg bud contains no clonally identifiable myoblasts (Bonner and Hauschka, 1974) but, after 6–11 days of growth on the CAM in the presence or absence of nerves, fully 23% of the clonable cells are myogenic. Thus, a portion of the total clonable myoblast population can arise from nondifferentiated cells in the absence of nerves. This agrees with the generally accepted notion that myogenesis is initially nerve independent.

Two broad categories of muscle-colony-forming cells have been described, conditioned-medium-requiring (CMR) and fresh-medium-sufficient (FMS) (Hauschka and White, 1972,; White *et al.*, 1975). CMR and FMS are subsets of the same clonable myoblast population and are distinguished on the basis of their culture-medium requirements for differentiation; FMS myoblasts will differentiate in both fresh and conditioned medium while CMR myoblasts will differentiate only in conditioned medium. The CMR cell level is calculated by substracting the percent muscle colony differentiation in fresh mediu from that in conditioned medium. Whe the data of Table 1 are examined in th light of these different cell types it can b seen that, in the case of innervated stag 21–22 leg bud grafts grown for 8 to 10 day (rows C and F), the FMS population in creases from 13 to 29% ($P < 0.01$), whi the CMR population increases from 15 (2 minus 13) to only 22% (51 minus 29), a insignificant increase considering the van ation in the data. The increase of musc colony differentiation in conditioned me dium is due almost entirely to an increas in the FMS population. These data ar consistent with the hypothesis that the e fects of innervation are reflected by larg relative increases in the FMS cell popula tion and perhaps by smaller increases i the CMR population.

While no differences in growth, morpho ogy, and cell number between innervate and noninnervated grafts have been ob served, there are obvious differences i these parameters between *normal* an grafted legs. Thus, the absolute values o the numbers reported in Table 1 do no relate to those of normal muscle of compa rable age but do suggest that a simila innervation-dependent myoblast popula tion should be demonstrable in normall developing embryonic leg tissue.

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DEVELOPMENTAL BIOLOGY 47, 228–232 (1975)

Insulin Binding to Cells of Several Tissues of the Early Chick Embryo

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The binding of I^{125}-labeled insulin to isolated cells from several tissues of the 3- and 4-day chick embryo was determined over a concentration range of insulin from 2×10^{-11} to 2×10^{-7} *M*. The cells were obtained from limb bud and nonlimb bud tissues of the 4-day chick, from the headless 3-day chick embryo, and from cartilage of the 12-day embryo. The amount of bound insulin was found to be similar for the cells from the different embryonic tissues. Some implications of these findings for the interpretation of the nature of the binding sites and the teratogenic effect of insulin are discussed.

INTRODUCTION

Deviations from normal embryonic development occur under many experimental conditions (11). One line of investigations aiming at the understanding of teratogenic forms of development had its beginning in the observation of the effects of insulin on the early development of skeletal appendages (17, 20). Work based on this original observation has branched out in several directions and from its results certain inferences were made about the possible mechanisms of insulin action, e.g., interference with metabolic reactions that depend on nicotinamide as cofactor (18, 19, 21, 22, 24). However, the question of why the development of some and not of other organ primordia is affected by insulin has remained unexplored. It seemed desirable, therefore to obtain information whether this difference in the teratogenic activity of insulin is related to corresponding differences in the binding of insulin to cell surfaces, the first step in the interaction of insulin with the cell. In the past, such work did not seem promising because of the inadequacies of available methods. With the elaboration of procedures for the analysis of the initial interaction between insulin with cell surface components (4) it has become possible to compare binding of insulin to tissues that are affected by insulin and those that are not. From the data reported here it is apparent that cells from limb buds (4-day embryo), from nonlimb bud tissues (3- and 4-day embryos), and from differentiated cartilage (11-day embryos) bind similar amounts of insulin. At the same time it became apparent that the levels of bound insulin may depend upon cell interactions that take place during cell aggregation and hence during organization of cells in tissues. Therefore the possibility cannot be excluded, at the present time, that, although no differences in the insulin binding of cells in the isolated state could be detected, such differences may still exist in cells in different states of organization.

MATERIALS AND METHODS

Determinations of binding of insulin per cell were carried out with cell preparations from four tissue types: whole bodies (without heads) from 3-day chick embryos; mesenchyme dissected from 4-day embryo limb buds; whole body without head and without limb buds from the same embryos; and thigh cartilage from 12-day chick embryos.

After dissection, the tissues from 1- to 4-

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dozen embryos were digested in 5 ml of collagenase (2 mg/ml; Worthington Biochemicals) in 0.1% BSA (bovine serum albumin), 0.1 *M* $MgSO_4$, 0.05 *M* Tris–HCl (pH 7.4 at 25°C) at 37°C for 15 min, then in 10 ml of collagenase for 45 min. The tissues were washed three times with buffer and made into a single-cell suspension by pipetting through a narrow-opening Pasteur pipet in 1% nutrient mixture F-10 (Gibco), 15 m*M* HEPES-TES (pH 7.4 at 25°C). Cells were pelleted in a refrigerated IEC centrifuge at 1500 rpm for 3 min and resuspended in BSA. To permit recovery of the cells from possible damage after collagenase digestion and mechanical dispersion, the above suspension was left for 2 hr at 37°C with swirling every 10 min to prevent aggregation of cells. After regeneration the cells were pelleted and resuspended in appropriate volumes of 1% BSA, and cell counts were then made.

Incubation with I^{125}-labeled insulin was carried out in a total volume of 100 or 500 μl using 8×10^5 or 2.5×10^6 cells and varying amounts of I^{125}-insulin (Cambridge Nuclear) all in 1% BSA solution. In order to determine nonspecific I^{125}-insulin-binding to cells an excessive amount (8 μg) of native insulin was added. Cells were incubated overnight at 4°C, then washed by centrifuging three times with 0.1% BSA at 4°C. Cells were counted for bound I^{125}-insulin in 0.5 ml of water and 4 ml of Aquasol (New England Nuclear) or Handifluor (Mallinckrodt) in a #300 Isocap (Searle Analytic) scintillation counter at 55% efficiency.

RESULTS

Cells from 4-day headless chick embryos were incubated to equilibrium with varying concentrations of I^{125}-insulin (Fig. 1). In all experiments I^{125}-insulin was bound to the cells. In most experiments the range over which insulin binding rapidly increased was 2×10^{-10}–7×10^{-8} *M*. A similar concentration dependence was observed by Kahn *et al.* (15) for rat liver membranes and by Gavin *et al.* (10) for human lymphocytes. Cuatrecasas (3) reported concentrations on the lower end of this range for rat adipocytes.

The maximal levels (at and above 7×10^{-8} *M*) of insulin bound per cell are of the same order of magnitude in the cell preparations from the different tested organ primordia. Table 1 shows that 4-day chick

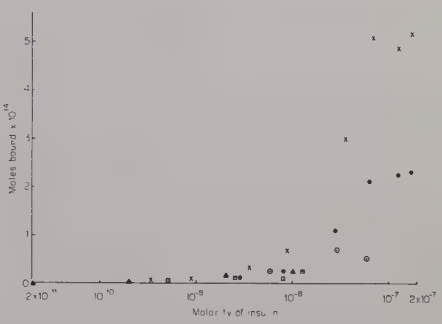

FIG. 1. Binding of insulin (ordinate) to cells of 4-day embryos as function of insulin concentration (abscissa). Experiment 1 (⊙), 8×10^5 cells/sample. Experiment 2 (▲), 4×10^6 cells/sample. Experiment 3 (⊡), 8.5×10^5 cells/sample. Experiment 4 (×), 2.4×10^6 (aggregating) cells/sample. Experiment 5 (●), 2.5×10^6 cells/sample. Incubation volume was 0.1 ml for Experiments 1 and 3–5; 0.5 ml for Experiment 2.

TABLE 1

NUMBER OF RECEPTOR SITES PER CELL FOR VARIOUS CELL TYPES[a]

Source of cells	Number of insulin binding sites per cell
Headless whole body (4 day)	11,700
Headless whole body (4 day) without limb buds	12,200
Cartilage (12 day)	15,800
Limb bud (4 day)	9,300
Adipose tissue	11,000
Cultured lymphocytes	12,850
Circulating lymphocytes	2,050

[a] The values for chick embryo tissues are calculated from data obtained at an insulin concentration of 1.6×10^{-7} *M*; value for adipose tissue from rat adipocytes from Ref. (3); values for lymphocytes from Ref. (9).

embryos with and without limb buds, 4-day chick embryo limb buds, and 12-day chick cartilage leaflets all have from 9300 to 15,800 insulin-binding sites (IBS) per cell. Four-day limb buds seem to have slightly lower insulin-binding capacity (9300 IBS) than the rest of the embryo at that time (12,800 IBS), although the difference is of no statistical significance. These values compare closely to values of 11,000 IBS per rat adipocyte reported by Cuatrecasas (3). Gavin reported 12,850 IBS for cultured human lymphocytes and 2050 IBS for circulating human lymphocytes (9).

Not unlike the difference Gavin found between cultured and circulating lymphocytes, we found that the moles of insulin bound per cell varied according to the concentrations of cells in the incubating solution. Experiment 1, Fig. 1, had a concentration of cells lower by a factor of 3 than in Experiments 4–5. Correspondingly, in Experiment 1, the moles of insulin bound per million cells at saturation was much lower than in Experiments 4–7. In Experiment 4 the cells were left to aggregate for three-fourths of the 2-hr recovery step rather than dispersed every 10 min. Here the insulin bound was double that in Experiment 5 where conditions were otherwise nearly the same.

A Scatchard plot of the results given by Experiment 4 gives a typical biphasic curve (Fig. 2) (25). The usual interpretation of this type of curve assumes the presence of independent receptor sites with high and low affinity for insulin (9, 15). However, negative cooperative binding has been proposed as an alternative interpretation (5, 15) with the affinities determined by receptor site interaction. According to this interpretation, deviations regarded as scatter in a Scatchard plot could be real. This may be the case with the high value for the ratio of bound to free insulin near 3.5×10^{-8} M incubation concentration of insulin which was observed in several other experiments (data not shown) as well as the rapid decline in the ratio of

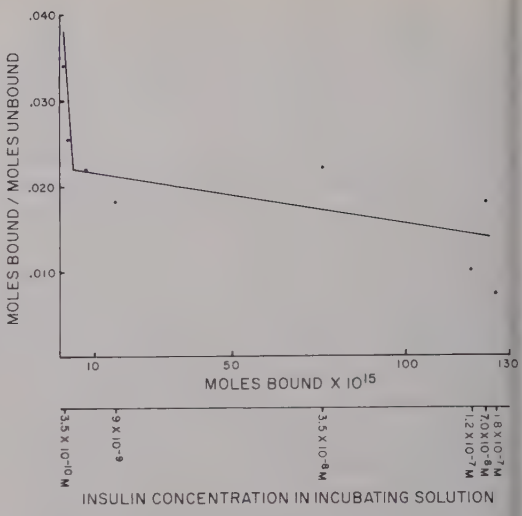

FIG. 2. Plot of bound/free I^{125}-labeled insulin as function of insulin bound to cells of 4-day chick embryo (Scatchard plot). The values below 5×10^{-15} mol indicate the high affinity region, the values above 5×10^{-15} the low affinity region. Lines were obtained by least-squares method.

bound to free insulin above incubation concentrations of 7×10^{-8} M insulin. The latter values would indicate that the insulin-binding sites are saturated at that and higher insulin concentrations.

DISCUSSION

In discussing the reported results the term insulin receptor sites is used to designate the cell surface groups that bind insulin. It is not known whether these sites in the embryo bind specifically only insulin or can bind other hormones or inactive macromolecules as well. Also the proof for binding of insulin solely to the cell surface has recently been questioned (16).

The observed binding of insulin to cells of several organ primordia of the early chick embryo opens the question of the nature of the binding sites and of their possible functional significance. Until recently, it was thought that insulin was not present in the chick embryo before the 10th day of development (12) and that, therefore, appearance of receptors preceded production of the hormone. Such a relationship has been demonstrated to

hold for glucocorticoid receptors in the developing retina (23). However, recent tests (2) demonstrated the presence of insulin in both the pancreas and the serum of 4-day-old embryos.

In this context, it is of some interest to compare the levels of insulin that are used to bring about *in vivo* teratogenic effects and to demonstrate binding to the cells of the embryo with the concentrations of insulin in the serum of the developing embryo. The observed serum levels in the embryo (2) correspond to a concentration of about 5×10^{-11} *M*. In the tests reported here, binding was observed at insulin concentrations above 10^{-9} *M*, reaching maximal levels at 10^{-8} *M*. Teratogenic effects are observed at insulin concentrations of about 1×10^{-6} *M* in the yolk and also in the semisolid nutrient agar (13) used for experiments with chick embryo explants. It is likely that, at least in case of the explants, only a small part of the insulin present in the agar medium becomes available to the embryo since immersion of the embryo into liquid medium elicits about a 100-fold increase in the response to insulin (13). Similarly, injection of insulin into the yolk may lead to an equally low uptake of the hormone. If this is the case the levels that lead to measurable binding and *in vivo* teratogenic effects would be similar. On the other hand, the levels in the serum of the embryo, or even of the adult, seem to be considerably lower than the concentrations needed for demonstration of readily measurable binding by isolated cells. Insulin at the low physiological serum concentration would be bound by the high affinity receptors at a higher proportion than at insulin concentrations used for binding studies and production *in vivo* of teratogenic effects.

The divergence in the concentrations of insulin that are found normally in the serum of embryos and mature animals and the much higher concentrations required for the production *in vivo* of teratogenic effects raises the question as to the possible mechanism of the teratogenic insulin effect. It could be proposed that insulin at high concentrations and concomitant binding to low affinity receptors have an injurious effect on some cell types and not on others. Alternatively, one could consider that the insulin which binds at the higher concentrations to the low affinity receptors actually interferes with the use of structurally related but functionally different hormones. Similarities have been observed in certain amino acid sequences of nerve growth factor and of insulin (6, 7), but nerve growth factor does not interfere with insulin binding (1), and insulin only slightly inhibits binding of nerve growth factor (8). On the other hand somatomedin, which is essential for sulfation of glycans, very readily displaces insulin from its receptors (14). If a hormone like somatomedin is present in early chick embryos, its displacement by insulin could be a teratogenic mechanism for insulin effects, in particular in primordia which are precursors of cartilagenous tissues in which sulfation occurs during early stages of development.

In discussing the meaning of the observed levels of bound insulin the following point has to be considered. The data obtained in Experiment 4 (Fig. 1) suggest that cells in the process of aggregation bind more insulin per cell than cells that are prevented from interaction with each other. Therefore, results obtained with isolated cells cannot be directly used for comparison with cells in tissues. It follows that the similarity in the binding of insulin by isolated cells from primordia with different sensitivity to the teratogenic action of insulin does not exclude the existence of differences in insulin binding by the cells in the respective organized tissue. This problem could be approached by following the changes in the insulin-binding capacity of cells at increasing degrees of interaction, e.g., at different stages of histiotypic aggregation.

Also, it remains undecided at the pres-

ent time whether the small amounts of insulin that are bound to cells from different tissues at low insulin concentration (high affinity receptors) are equally similar to those found for the total bound insulin.

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DEVELOPMENTAL BIOLOGY 47, 233–235 (1975)

Enhanced Cell Aggregation in *Dictyostelium discoideum* by ATP Activation of Cylic AMP Receptors

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Accepted June 23, 1975

Cell aggregation in the cellular slime mold *Dictyostelium discoideum* is mediated by cyclic AMP. In the presence of ATP the onset of cell aggregation is enhanced and cyclic AMP receptors are activated. The number of phosphorylation sites is species dependent, and two main phosphorylated proteins with MW of, respectively, 20,000 and 15,000 are localized.

INTRODUCTION

Cyclic AMP induces the multicellular phase in the larger species of *Dictyostelium* (7). Cyclic AMP action on the single amoeba is extracellular (11), specific (5) and probably mediated by a cell-membrane-bound receptor (9, 10). The binding of cyclic AMP is limited to those species using this cyclic nucleotide as chemotactic agent (10). This article reports the earlier cell aggregation and cyclic AMP receptor activation in the presence of ATP and its possible mechanism via cell-membrane phosphorylation by an endogenous cyclic AMP-independent protein kinase.

MATERIALS AND METHODS

Amoebae were grown and harvested as described previously (6). Cell aggregation was studied using small populations of amoebae on hydrophobic agar (6). Cyclic AMP binding was measured at 22°C on Millipore filters (10). Cells were incubated with 50 μM [γ-^{32}P]ATP (100 mCi/mmol, Amersham) and the phosphorylated proteins were separated from free [γ-^{32}P]ATP by repetitive washing with NaOH and ice-cold trichloroacetic acid (TCA) precipitation or by solubilization in 1% sodium dodecyl sulfate (SDS) containing 40 mM dithiothreitol and 1 mM EDTA and subsequent electrophoresis (3) after incubation at 80°C for 30 min. Appropriate controls were treated the same way.

RESULTS AND DISCUSSION

When small drops of an amoebal suspension of *Dictyostelium discoideum* were placed on hydrophobic agar plates containing 2 mM ATP, disodium salt, and 2 mM $MgSO_4$, aggregation started about 3–5 hr earlier than in control plates containing 2 mM $MgSO_4$ and 4 mM NaCl. Aggregation started in control plates about 10 hr after plating of the amoebae. Substitution of ATP by ADP or AMP did not affect the time of the onset of aggregation. Preaggregative amoebae became phosphorylated when incubated with [γ-^{32}P]ATP (Fig. 1), the precipitable radioactivity being stable in hot HCl but not in hot NaOH. Cyclic AMP in concentrations ranging from 10^{-9} to 10^{-6} M was without effect on the rate of amoebal phosphorylation. Cyclic AMP-independent protein kinase assayable with exogenous substrate has also been reported by Weinstein and Koritz (12). The lack of endogenous phosphorylation found by these authors might be explained by their measurement of phosphorylation in 10^6 cells at lower [γ-^{32}P]ATP specific activity (20 mCi/mmol) as well as to differences between strains.

By SDS electrophoresis it was shown that two major species with molecular weights of, respectively, about 20,000 and 15,000 were preferentially phosphorylated (Fig. 2).

Earlier cell aggregation needs to be ac-

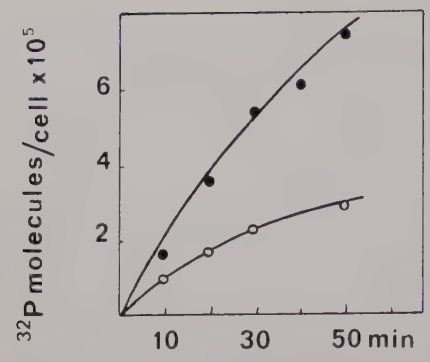

FIG. 1. Time course of phosphorylation in *D. discoideum* (●) and *D. mucoroides* (○). Phosphorylation was carried out in a final volume of 200 μl containing 50 μ*M* [γ-^{32}P]ATP, 10 m*M* NaF, 10 m*M* $MgSO_4$ and 10^7 cells in a 1% saline solution, pH 7. After different incubation times at 20°C the reaction was stopped by adding 200 μl of ice-cold 10% TCA. After centrifugation the pellet was dissolved in 100 μl of 1 *N* NaOH and precipitated again by adding 1 ml of ice-cold 5% TCA. This step was repeated twice. The final pellet was washed twice with 5% TCA and dissolved in NaOH, and the Cerenkov radiation was measured.

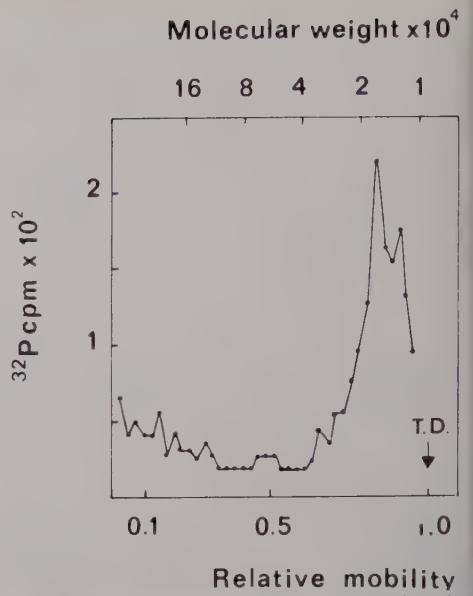

FIG. 2. Electropherogram of phosphorylated amoebae of *D. discoideum*. Amoebae (2×10^7 cells) were incubated as described in Fig. 1. After 30-min incubation at 20°C, the reaction was stopped with SDS as described in Materials and Methods. After electrophoresis, gels were cut into 1.5-mm slices and the Cerenkov radiation measured in 8 ml of water. Hemoglobin, trypsin and bovine serum albumin were used as standards for the molecular weight determination. T.D., tracking dye.

companied by earlier cyclic AMP binding and thus, when the cyclic AMP binding activity was measured in the presence of 1 m*M* ATP and 1 m*M* $MgSO_4$, this activity was maximal about 4 hr earlier than in controls (Fig. 3).

Cyclic AMP secretion increases at the onset of aggregation (1) and therefore earlier aggregation should be accompanied by earlier increase in cyclic AMP secretion. However, this hypothesis needs to be proven. Whether any one of the two main phosphorylated species corresponds to the chemotactic receptor also needs further investigation. Autophosphorylation of cyclic AMP receptors by cyclic AMP-dependent protein kinase has been reported before (2, 4, 8), although a difference in cyclic AMP binding activity between phosphorylated and nonphosphorylated molecules was not demonstrated. In Fig. 1 it can be seen that the number of free phosphorylatable sites in *D. discoideum* is more than two times bigger than in *D. mucoroides*, which agrees with previously described differences in the number of cyclic AMP receptors per cell (10). However, whether in the

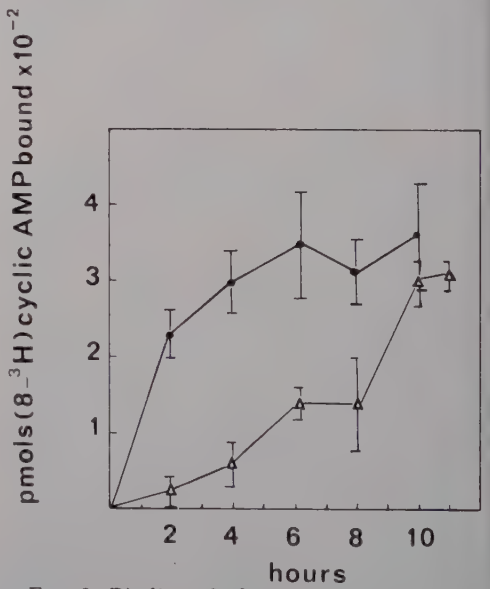

FIG. 3. Binding of 8-^{3}H-labeled cyclic AMP in *D. discoideum*. Cells (10^5) were deposited on Millipore filters and cyclic AMP binding determined, as described by Mato and Konijn (10), at different developmental stages in the absence (△) and presence (●) of 1 m*M* ATP.

preaggregative stage most of the phosphorylation sites are free in both species and therefore the results in Fig. 1 comparable or some of the phosphorylation sites have been already phosphorylated in *D. mucoroides*, which species aggregates earlier than *D. discoideum*, needs to be established.

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Ergogenic Aids and Muscular Performance

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